Vaginal cytokines do not differ between postmenopausal women with and without symptoms of vulvovaginal irritation

Petra Stute, MD, Zahraa Kollmann, MD, Nick Bersinger, PhD, Michael von Wolff, MD, Andrea R. Thurman, MD, and David F. Archer, MD

Abstract

Objective: The aim of this exploratory pilot study was to determine if there are differences in vaginal cytokine levels between postmenopausal women with and without vulvovaginal irritative symptoms (itching, burning, or pain).

Methods: Postmenopausal women (n = 34) not using hormone therapy and presenting with or without symptoms of vulvovaginal irritation were asked to volunteer for this study. Each participant underwent a vaginal examination and screening for vaginitis using Amsel criteria, pH, and light microscopy. A vaginal lavage with 5.0 mL of sterile saline was carried out, and a peripheral blood sample was obtained. The vaginal lavage and serum samples were assayed for interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α by specific enzyme-linked immunosorbent assays. Results were adjusted for total protein concentration and presented as the amount of cytokines per protein (pg/μg protein). Statistical analysis was performed using SAS version 9.3 (SAS Institute, Cary, NC). The means and SDs of all variables among women with and without vulvovaginal irritation were compared using independent-samples Student’s t test.

Results: A total of 26 postmenopausal women were enrolled into the study (symptomatic, n = 15; asymptomatic, n = 11). The mean (SD) vaginal pH for all participants was 5.9 (1.2). There were no significant differences (P > 0.05) in age, age at menopause, vaginal pH, and vaginal and serum cytokines and chemokines (IL-1β, IL-6, IL-8, and tumor necrosis factor-α) among symptomatic versus asymptomatic women. IL-8 was the most abundant vaginal cytokine, with mean (SD) vaginal IL-8 levels being 4.1 (3.4) and 3.1 (3.9) pg/μg protein in the symptomatic versus asymptomatic groups, respectively (P = 0.55). There were no significant linear correlations (P > 0.05) between serum and vaginal cytokine levels for all endpoints.

Conclusions: The presence or absence of postmenopausal vulvovaginal symptoms does not significantly differentiate vaginal inflammatory markers. Serum and vaginal cytokines are not significantly linearly correlated among postmenopausal women with and without symptoms commonly associated with vaginal atrophy, implying that this is a local reaction.

Key Words: Vulvovaginal atrophy – Menopause – Interleukin-1β – Interleukin-6 – Interleukin-8 – Tumor necrosis factor-α.

Vulvovaginal irritation is a frequent complaint among postmenopausal women who are not using hormone therapy (HT). Common symptoms of vaginal atrophy include dryness, itching, burning, and dyspareunia. Clinical improvement in these symptoms is seen with estrogen therapy. Thus, most clinicians attribute these symptoms to estrogen deficiency, but the underlying pathophysiology of this clinical symptom complex is not well defined.

The symptoms of postmenopausal vaginal atrophy are similar to those of yeast vaginitis. A number of animal and human studies have shown that the levels of cytokines in cervicovaginal lavage positively correlate with the histopathology and clinical signs of vaginal inflammation.

Significant effort is being exerted to identify the biomarkers of vaginal epithelial/mucosal damage and safety. These studies have been used to investigate topical microbicide products—used to prevent sexually transmitted diseases—that preserve or have limited effects on natural immune barriers. The proinflammatory interleukins (ILs) IL-1α, IL-1β, and IL-8, as well as the anti-inflammatory protein IL-1 receptor antagonist, have emerged as such biomarkers.

Estrogen has been shown to influence cytokine activity in the reproductive tract involving ovulation, menstruation, and
The impact of endogenous and exogenous Neisseria gonorrhoeae and Cervical IL-1β and IL-10 levels, for example, both peak just before ovulation in natural cycles, and cervical mucus IL-1β levels were found to correlate directly with changes in estrogen levels during a natural cycle. Similarly, cervicovaginal IL-1β and IL-6 levels were found to vary throughout the ovulatory cycle, with both IL-1β and IL-6 being fivefold higher in the follicular phase than in the luteal phase.

Vaginal cytokines have not been studied as extensively in postmenopausal women and not at all in women with or without symptoms secondary to vaginal atrophy.

Our hypothesis is that postmenopausal vulvovaginal symptoms are associated with elevated vaginal cytokine levels, similar to inflammation. The principal goal of our study was to determine if differences in vaginal and serum cytokine levels exist between postmenopausal women based on the presence or absence of vulvovaginal symptoms. Plasma cytokine levels were assessed concurrently and compared with vaginal levels to ensure that the vaginal levels obtained did not result from contamination by plasma transudate and to determine if there were any correlations between vaginal and systemic cytokine levels.

METHODS

Study population
The study population consisted of 34 postmenopausal women who were recruited from the menopause center at the Inselspital Berne (Berne, Switzerland). Symptoms of vulvovaginal irritation were defined as pruritus, burning, discharge, and/or dryness. Participants were classified as being either symptomatic or asymptomatic using the Menopause Rating Scale II.

Women were eligible to participate in the study if they were 40 years or older, had a history of at least 1 year of amenorrhea, had normal Papanicolaou test results within the past year, and agreed to abstain from sexual intercourse and use of over-the-counter vaginal products for 7 days before the collection of vaginal samples. Criteria for exclusion were as follows: administration of any form of oral HT within 2 months of entry into the study or administration of any form of transdermal or vaginal HT therapy within 1 month of entry into the study; active vulvovaginal candidiasis, Trichomonas vaginalis, or bacterial vaginosis based on light microscopy of vaginal secretions, vaginal pH, whiff test, and KOH preparation; untreated cervical, vaginal, or vulvar intraepithelial neoplasia; active sexually transmitted infections, including herpes simplex virus, gonorrhea, and Chlamydia trachomatis; use of pessary; active treatment with tamoxifen, raloxifene, or other forms of selective estrogen receptor modulators; undiagnosed vaginal bleeding; hysterecomy; history of or current breast cancer, endometrial cancer, or endometrial hyperplasia; hypertiglyceridemia or venous thromboembolism; and immunocompromised women, including those with human immunodeficiency virus (HIV) infection, on long-term glucocorticoid use, or on active treatment with immunosuppressive agents.

Study procedures
This was a single-center nonintervention study of postmenopausal women with or without symptoms of vulvovaginal irritation who presented for assessment of vaginal and serum cytokine content. The study was approved by the local ethics committee (KEK no. 58/10; ClinTrials.gov identifier NCT 01899612). All women provided an informed consent form for participation in this trial. At screening (visit 1), medical history was taken, and a gynecologic examination (including assessment of vaginal pH, saline and KOH preparations, and whiff test) was performed to test for bacterial vaginosis (Amsel criteria), vaginal yeast, and Trichomonas vaginalis (microscopy). Women were also screened for sexually transmitted infections, including C. trachomatis and Neisseria gonorrhoeae, and were not eligible if they tested positive for any of these infections. Enrollment investigations (visit 2) were performed within a maximum of 2 weeks after visit 1 and included another speculum examination for vaginal pH, light microscopy of vaginal secretions, whiff test, and vaginal lavage (VL). In addition, 10 mL of peripheral venous blood was obtained and centrifuged for 15 minutes at 1,200 × g, and the serum was stored at −34°C for the quantification of cytokine, estradiol, and lipid levels.

Cytokine evaluation
VL specimens were collected at the enrollment visit (visit 2). VL samples were obtained by flushing the lateral, anterior, and posterior walls and fornice of the vagina with 5 mL of sterile phosphate-buffered saline using a sterile plastic transfer pipette. As much of this fluid as possible was collected after 30 seconds. The sample was immediately placed on ice and centrifuged at 800g for 10 minutes to remove the cellular components and debris. The supernatant was aspirated and stored in aliquots at −34°C for subsequent analysis by enzyme-linked immunosorbent assay (ELISA). The sediment was re-suspended in 1 mL of physiological saline, and leukocyte counts were obtained by hemocytometry. All samples were tested with Hemastix (no. 2816; Ames, Elkart, IN), which can detect 1.5 mg of hemoglobin per milliliter of fluid, to rule out any significant contamination with blood. Samples that contained red blood cells were eliminated. The VL samples were tested in duplicate for the cytokines IL-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) using commercially available, validated manual microplate ELISA kits (Quantikine; R&D Systems, Oxford, England) according to the manufacturer’s instructions. The functional sensitivities of these assays, as established for serum, were 1, 0.7, 7.5, and 0.2 pg/mL for IL-1β, IL-6, IL-8, and TNF-α, respectively. The intra-assay and interassay coefficients of variation at concentrations similar to the ones encountered in our samples (Table 3) were 3.3%, 4.2%, 6.5%, and 5.2%, and 8.4%, 6.4%, 6.1%, and 7.4% for the above four cytokines, respectively. The total protein concentration in the VL was determined with the QuantiPro concentration in the VL was determined with the QuantiPro

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Assay</th>
<th>Sensitivity (pg/mL)</th>
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</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>TNF-α</td>
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<td>0.2</td>
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The results were normalized for the total protein concentration of each component.
Cytokines in Women with Vulvovaginal Symptoms

Factors associated with cytokine levels
Mean age, age at menopause, time since menopause, prior use of systemic or topical HT, presence or absence of symptoms of vulvovaginal irritation, serum estradiol levels (ECLIA, Roche Modular E170), serum lipid levels (total cholesterol, low-density lipoprotein [LDL] cholesterol, high-density lipoprotein [HDL] cholesterol, and triglycerides; Roche, Modular P800), and serum IL-1β, IL-6, IL-8, and TNF-α levels (Quantikine; R&D Systems) were studied in relationship to vaginal IL-1β, IL-6, IL-8, and TNF-α levels. The comparison of serum to vaginal cytokines was performed to see if any correlation between the values would suggest that the vaginal cytokines originated from the serum.

Statistical analysis
Statistical analysis was performed using SAS version 9.3 (SAS Institute, Cary, NC). Normal distribution of all endpoints was confirmed before comparison using Student’s t test. Equality of variances in the means was checked for each comparison, and either a pooled P value (for equal variance) or a Satterthwaite P value (for unequal variance) was reported. R and P values were calculated for the comparison of vaginal and serum cytokines. Categorical variables were compared using Fisher’s exact test. The means and SDs of all variables among women with and without vulvovaginal irritation were compared using independent-samples t test.

RESULTS
Participants’ characteristics
A total of 26 postmenopausal women were enrolled into the study. Participants’ characteristics are presented in Table 1. Mean vaginal pH corresponded to severe vulvovaginal atrophy.25 Screening for bacterial vaginosis yielded positive results in 2 of 15 (13%) symptomatic women and in 2 of 11 asymptomatic women (18%). VL was performed in those women after standard treatment and negative retest results. Serum estradiol levels fell within the postmenopausal range for all participants (n = 25; mean [SD], 16.0 [22.9] pmol/L), with no differences between symptomatic (21.0 [23.1]) and asymptomatic (12.3 [22.4]) women (P = 0.35). The mean (SD) baseline serum lipid levels (n = 25) were as follows: total cholesterol, 5.9 (1.1) mmol/L; LDL cholesterol, 3.4 (0.9) mmol/L; HDL cholesterol, 1.8 (0.5) mmol/L; triglycerides, 1.3 (0.5) mmol/L.

Table 3. Vaginal cytokine levels in postmenopausal women with and without symptoms of vulvovaginal atrophy

Vaginal cytokine levels were not significantly different between the symptomatic and asymptomatic women. IL-8 was the most abundant cytokine in serum. Mean serum levels of IL-1β, IL-6, IL-8, and TNF-α were not significantly different between the two groups (Table 2). The serum cytokine levels were not linearly correlated with time since menopause or serum estradiol levels.

Vaginal leukocytes and cytokines
The leukocyte counts in VL pellets were 6.1 (13.5) × 10⁶ for symptomatic women and 7.5 (17.7) × 10⁶ for asymptomatic women (P = 0.83). Vaginal IL-1β, IL-6, and IL-8 levels were available for 73% of symptomatic women and 82% of asymptomatic women. Vaginal TNF-α levels were available for 40% of symptomatic women and 36% of asymptomatic women. Vaginal IL-8 cytokines, similar to serum cytokines, were the most abundant vaginal cytokines (Table 3). The vaginal concentrations of IL-1β, IL-6, and TNF-α were 4%, 2.5%, and 0.4% of vaginal IL-8 levels, respectively. Overall, there were no significant differences in vaginal cytokine levels between groups, but there were wide ranges in individual values (Table 3). The vaginal cytokine levels were not correlated with time since menopause and serum estradiol levels. When comparing women who were treated for bacterial vaginosis before VL (n = 4) with women who had negative test results for infectious vaginitis (n = 22) at screening, no significant

Table 2. Serum cytokine levels in postmenopausal women with and without symptoms of vulvovaginal atrophy

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Symptomatic group</th>
<th>Asymptomatic group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β, pg/mL</td>
<td>16.81 (14.05) [10]</td>
<td>5.66 (9.34) [8]</td>
<td>0.07</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>4.20 (3.82) [10]</td>
<td>2.29 (1.54) [8]</td>
<td>0.17</td>
</tr>
<tr>
<td>IL-8, pg/mL</td>
<td>58.69 (90.22) [10]</td>
<td>11.21 (10.17) [8]</td>
<td>0.13</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>8.66 (8.41) [5]</td>
<td>2.86 (2.01) [4]</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD) [n].
TABLE 4. Vaginal cytokine levels per protein content in postmenopausal women with and without symptoms of vulvovaginal atrophy

<table>
<thead>
<tr>
<th>Vaginal cytokine per protein content</th>
<th>Symptomatic group</th>
<th>Asymptomatic group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β, pg/µg protein</td>
<td>0.21 (0.14) [11]</td>
<td>0.20 (0.26) [9]</td>
<td>0.95</td>
</tr>
<tr>
<td>IL-6, pg/µg protein</td>
<td>0.02 (0.01) [11]</td>
<td>0.08 (0.18) [9]</td>
<td>0.31</td>
</tr>
<tr>
<td>IL-8, pg/µg protein</td>
<td>4.07 (3.37) [11]</td>
<td>3.62 (4.02) [9]</td>
<td>0.79</td>
</tr>
<tr>
<td>TNF-α, pg/µg protein</td>
<td>0.02 (0.01) [6]</td>
<td>0.04 (0.05) [4]</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD) [n].

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The mean vaginal IL-1β ranged between 3.7 (20) and 125 pg/mL, respectively. Only one study measured vaginal IL-6 levels under these conditions, with mean absolute values ranging from 1 pg/mL (luteal phase of the menstrual cycle) to 6 pg/mL (follicular phase of the menstrual cycle). The mean vaginal IL-8 ranged from 180 (20) to 946 pg/mL. In general, all but one study found vaginal TNF-α levels to be close to or below the detection limit. The mean absolute values for vaginal IL-1β, IL-6, IL-8, and TNF-α in postmenopausal women in our study fell within the ranges given above for premenopausal women. Similar to our study, IL-8 was the most abundant vaginal cytokine in previous trials. IL-8 is a potent chemotactic and activating factor for neutrophils and is activated by several innate factors such as IL-1. Accordingly, vaginal IL-1β positively correlated with IL-8 in our study.

Similar to our study, only three studies compared vaginal cytokines to serum cytokines. Comparable to our findings, there was no correlation between vaginal and serum cytokine levels.

The number of leukocytes and vaginal cytokines in our study did not differ between postmenopausal women with and without symptoms of vaginal atrophy. This finding contradicts our hypothesis that postmenopausal vulvovaginal symptoms are associated with elevated vaginal cytokine levels, similar to inflammation. According to previous studies, vaginal irritation induced by microbicides, local vaginal infection, and systemic infections such as HIV induce a proinflammatory response in the vagina that might weaken the natural mucosal immune barrier against sexually transmitted diseases. However, the failure to find differences in vaginal cytokines between symptomatic and asymptomatic postmenopausal women with symptoms associated with vaginal atrophy might be attributed
differences in participants’ characteristics, serum cytokines, vaginal leukocytes and cytokines, and VL protein content were found.

The VL protein content was 195.0 (156.0) µg/mL in symptomatic women and 295.7 (295.0) µg/mL in asymptomatic women (P = 0.27). Overall, vaginal cytokine levels per protein content did not reveal significant group differences (Table 4). Similar to absolute vaginal cytokine levels, vaginal cytokine levels per protein did not correlate significantly with time since menopause and serum estradiol levels. Expressing vaginal cytokine levels per milligram of vaginal protein reduced the large interindividual variation observed when cytokines were expressed as milligrams per milliliter.

Correlation analysis

Serum and vaginal cytokines were not correlated (all P > 0.05), with r values of −0.14, −0.07, 0.12, and −0.08 for IL-1β, IL-6, IL-8, and TNF-α, respectively. Similarly, there was no correlation between vaginal cytokines and vaginal leukocyte counts. However, vaginal IL-1β was significantly correlated with vaginal IL-8 (r = 0.58, P = 0.0068) and vaginal TNF-α (r = 0.79, P = 0.006). Vaginal IL-6 was significantly correlated with vaginal IL-8 (r = 0.61, P = 0.0041).
to the small sample size, a still-too-insensitive method for detecting subtle differences between VL samples, or symptoms that are limited to the vulva and introital tissues, and not to the vagina, and would thus escape VL assessment. Furthermore, because we did not compare our cohort with healthy premenopausal and postmenopausal women using HT, we actually do not know if the vaginal cytokine levels measured in our study would be higher, equal to, or lower than the levels in women exposed to endogenous or exogenous estrogens. Estrogens have been shown to restore the Lactobacillus microbiota in the vagina and thus to reduce the incidence of bacterial vaginosis.38 Bacterial vaginosis is thought to increase the susceptibility to HIV and other sexually transmitted diseases.39,40 Oral estrogens have been shown to down-regulate proinflammatory IL-1β and IL-6 gene expressions in the vagina.33 However, women in the control group in this study were not differentiated for symptoms of vaginal atrophy. Therefore, vaginal cytokine assessment in a larger symptomatic or asymptomatic postmenopausal cohort, using a standardized sensitive method with parallel assessment of the vulvar epithelium, is warranted.

CONCLUSIONS

Postmenopausal vulvovaginal symptoms do not correlate with vaginal inflammatory markers. There is no difference in serum or vaginal cytokines between postmenopausal women with and postmenopausal women without symptoms commonly associated with vaginal atrophy, implying that this is a local reaction. The clinical symptoms of vulvovaginal atrophy do not seem to reflect vaginal cytokines.

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