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The Effects of Reproductive Hormones on the Physical Properties of Cervicovaginal Fluid

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Abstract

Objectives—The purpose of this study was to determine the impact of contraception, menopause and vaginal flora on the physical and biochemical properties of cervicovaginal fluid.

Study Design—Vaginal swabs, cervicovaginal fluid (CVF) and cervicovaginal lavage (CVL) were collected from a total of 165 healthy asymptomatic women including: post-menopausal women (n=29), women in the proliferative (n=26) or follicular (n=27) phase, and women using the levonogestrel intrauterine device (LNG-IUD) (n=28), depomedroxyprogesterone acetate (DMPA) (n=28) or combined oral contraceptives (OCs) (n=27). Vaginal smears were evaluated using the Nugent score. The osmolality, viscosity, density and pH of CVL samples were measured.

Results—CVL from postmenopausal women and women with abnormal vaginal flora was less viscous and had higher pH than premenopausal women and women with normal flora, respectively. Women using hormonal contraceptives had more viscous CVL as compared to premenopausal women not using hormonal contraceptives, but this increase in viscosity was mitigated in the presence of bacterial vaginosis. Women using DMPA had less total protein in the CVL as compared to women using the LNG-IUD, and had similar protein content when compared to postmenopausal women.

Conclusion—The differences in CVL protein content between DMPA and LNG-IUD suggest that type of progesterone and route of delivery impact the vaginal environment. Contraceptive hormone users had more viscous CVL than women not using contraceptives. However, the presence of bacterial vaginosis impacted both the pH and viscosity (regardless of hormonal contraceptive use), demonstrating that vaginal flora has a greater impact on the physical properties of cervicovaginal fluid than reproductive hormones.

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Keywords

Contraceptives; cervicovaginal fluid; bacterial vaginosis

Introduction

Cervicovaginal fluid (CVF) is comprised of transudate from the vaginal epithelium as well as cervical mucus and secretions from the uterus and fallopian tubes. The mucin gel layer which coats the vaginal epithelium is one of the first line defenses in protection against pathogens of the genital tract[1]. In order for sexually transmitted pathogens, such as HIV, to establish infection, they must penetrate the mucus layer and attach to receptors on target cells in the cervical or vaginal epithelium. In a recent study, CVF, collected using a catamenial cup, slowed the diffusion of HIV-1 particles more than 200-nm PEGylated beads, which was dependent on the presence of HIV-1 envelope proteins. This demonstrates an important protective interaction between the CVF and HIV-1 particle[2]. In another study, the movement of HIV-1 through the CVF has been reported to be significantly slower at a pH of 4 and more rapid when the CVF was buffered to a pH of 6[3]. Thus, the physical properties of the CVF impact how efficiently virus particles can traverse CVF and infect the vaginal or cervical epithelium. Decreased viscosity of CVF may render the mucin gel layer more permissive to penetration. Additionally, CVF serves as a carrier for a broad array of antimicrobial peptides[4] and proteins including lysozyme[5], lactoferrin[6], secretory leukocyte protease inhibitor (SLPI)[7, 8], and human beta-defensins[9]. Therefore, the protein content of the CVF is also a key component in the innate mucosal defense.

One mechanism of progestin-dependent contraceptive efficacy is believed to be thickening of cervical mucus and preventing the transport of sperm from the vagina into the uterus and fallopian tubes[10]. The quality of the cervical mucus is dependent on reproductive hormones. Without the use of exogenous hormones, the first half of the menstrual cycle is characterized by increased estradiol levels and an increased amount of cervical mucus that is thin and watery to allow sperm penetration. In the second half of the menstrual cycle, predominated by increased progesterone levels, the cervical mucus becomes scant in amount, thick and opaque[11]. Several studies have reported a thickening of cervical mucus associated with progestin-only contraceptive methods using subjective measures of cervical mucus quality such as ferning and spinbarkeit[12–15]. Progestins subjectively thicken the cervical mucus within the cervical canal. However, less is known about the impact of reproductive hormones on cervicovaginal fluid, which provide a protective barrier over the vaginal epithelium. In addition to affecting the physical properties of the CVF, reproductive hormones mediate the biochemical content. Specifically, immunoglobulins, human beta-defensins and SLPI are lowest at mid-cycle when estradiol levels are elevated[16–18].

The effects of exogenous reproductive hormones on CVF could have clinical consequences. A few prospective, well-controlled studies have linked progestin-only injectable use to increased HIV risk[19–22]. The effect of reproductive hormones on the physical or biochemical composition of the CVF has not been completely characterized, but changes in

the mucin gel layer covering the vaginal epithelium is one possible biological mechanism by which hormonal contraceptives could impact the risk of HIV acquisition.

Normal vaginal microbiota, characterized by a predominance of lactobacilli, is thought to be protective against sexually transmitted infection. The presence of bacterial vaginosis (BV) is associated with an increased risk of HIV acquisition in an HIV uninfected woman[23] as well as increased risk of HIV transmission by an HIV infected women to an HIV uninfected male partner[24]. Bacterial vaginosis is characterized by an overgrowth of anaerobic bacteria and a decreased colonization by *Lactobacillus* species. This overgrowth of anaerobic bacteria is associated with increased levels of bacterial proteases and glycosidase in CVF[25]. Women with bacterial vaginosis have higher levels of vaginal sialidases. Sialidases are considered virulence factor in bacterial vaginosis[26]; they clip the negatively-charged sialic acid residues from the terminal end of the mucin oligosaccharides. Sialidase residues protect the oligosaccharide and the protein back-bone of the mucin molecule from degradation by mucin-degrading enzymes. The negatively-charged mucin molecules keep a rigid structure and trap pathogens, preventing them from reaching the vaginal epithelium. Bacterial vaginosis may cause thinning of the mucin gel layer thus impeding the capacity of the CVF to serve as a barrier against HIV infection.

To date, there have been few studies that have investigated the impact of reproductive hormones and vaginal flora on the physical and biochemical properties of the CVF. In the present study, we collected both cervicovaginal fluid (CVF) using a catamenial cup as well as cervicovaginal lavage (CVL) by washing the vaginal vault with sterile normal saline. The primary aim of this study was to characterize the impact of reproductive hormones on the viscosity, pH, density, osmolality and protein content of CVF. Due to the small volume and technical difficulties associated with performance of assays with the CVF, assessment of the physical properties of the CVF samples was not feasible. Therefore, the viscosity, pH and osmolality and density were measured only in the CVL samples. Because epidemiologic studies have linked BV and exogenous contraceptive use to increased HIV susceptibility, we hypothesized that the use of contraceptives, phase on menstrual cycle, menopausal status, and vaginal flora will impact the physical properties and protein content of CVL.

Materials and Methods

Study Population

Following Institutional Review Board approval by the University of Pittsburgh, informed consent was obtained from healthy, asymptomatic, HIV-negative women who were either between 18–46 years of age or over the age of 50. We enrolled premenopausal women into the study who fell into the five following categories on the basis of contraceptive use by self-report: 1) not contracepting on days 1–14 of the menstrual cycle, 2) not contracepting on days 15–28 of the menstrual cycle, 3) using combined-oral contraceptive pills for at least 6 months, 4) using depot medroxyprogesterone acetate (DMPA) injections for at least 6 months, 5) using the levonorgestrel IUD for at least 1 month. A group of postmenopausal women was also recruited; menopause was defined as age greater than 50 years of age without any vaginal bleeding in the previous one year. Women were excluded from the study if they had been pregnant or breastfeeding within the last 90 days, had vaginal

symptoms or evidence of vaginitis on clinical exam, had used vaginally-applied products in the prior week, had used antibiotics in the two weeks prior, had undergone a hysterectomy, or had a positive rapid HIV test. Additionally, postmenopausal women taking exogenous estrogen were also excluded. None of the postmenopausal women reported taking supplements containing phytoestrogens.

Upon enrollment, demographic information, medical, gynecologic and sexual histories were collected from each participant. A vaginal swab for pH, wet mount microscopy and Gram stain were collected. The Instead™ catamenial cup was inserted into the vagina up to the cervix by the clinician and left in place for at least 45 minutes. The catamenial cup was removed and placed into a 50 cc conical vial for transport to the laboratory. The cervicovaginal fluid samples were centrifuged at 2,000 x g for 10 minutes. The protein laden material was removed and the volume was measured. Due to the small volumes and difficulty working with the CVF samples, we were unable to assess the physical properties of the CVF specimens. Then, these samples were stored at -70C for future study. For collection of the cervicovaginal lavage (CVL), 10 milliliters of sterile normal saline was placed into the vagina, a lavage was performed for 1 minute and placed into 15 milliliter conical vial with 100 microliters of protease inhibitor (Sigma-Aldrich). A cervical swab was collected for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* testing using the Aptima GenProbe test. Women found to have these pathogens or *Trichomonas vaginalis* by wet mount microscopy were excluded from the analysis. All samples were stored at -70C until they were thawed for immediate testing. The osmolality (milliosmoles/kilogram, mOsm/kg), viscosity (centipoise, cP) of each sample was measured in triplicate using the Advanced Instruments MicroOsmometer Model 3320 and the Cambridge MicroSample Viscometer, respectively. Density was calculated by determining the weight in triplicate of 0.5 milliliter of sample. pH was measured using a Mettler Toledo pH meter. The Gram-stained vaginal smear was evaluated using Nugent criteria[27]. Fisher's exact test, Student's t-test, and one-way analysis of variance with post-hoc comparisons made using Bonferroni's multiple comparisons procedure were used to assess statistical significance using IBM SPSS Statistical software version 20 (IBM Corp, Armonk, NY, USA).

Results

Demographic Characteristics

The demographic characteristics of the study population are summarized in Table 1. The ages of women in the premenopausal groups were not statistically different (mean of 29 years); while the postmenopausal women had a mean age of 56 years. The majority of women were white and nonsmoking, with the exception of women using DMPA, who were more likely to identify themselves as black ($p<0.001$) and to report tobacco use ($p=0.046$). Women using hormonal contraceptives reported less frequent condom use as compared to women not using hormonal contraceptives ($p<0.001$).

Effects of Reproductive Hormones and Bacterial Vaginosis

The physical properties of cervicovaginal lavage were different in postmenopausal women as compared to premenopausal women with respect to pH ($p<0.001$) and viscosity ($p<0.001$)

of CVL, but not osmolality or density (Table 2). The protein content of CVL was lower in postmenopausal women when compared to premenopausal women ($p < 0.001$), and all premenopausal groups had a higher protein content in the CVL as compared to postmenopausal women, with the exception of the women using DMPA (Figure 1). Women using the LNG-IUD had more CVL protein as compared to women using DMPA. No other differences in physical properties were detected between any of the premenopausal groups.

When all of the women using hormonal contraceptives (DMPA, COCs, and LNG-IUD) were grouped and compared to premenopausal women not using hormonal contraceptives, women with normal flora and using hormonal contraceptives were found to have higher viscosities compared to women not using hormonal contraceptives with normal flora (figure 2, left). The presence of bacterial vaginosis mitigated the increased CVL viscosity seen in women using hormonal contraceptives (figure 2, right).

Bacterial vaginosis had significant effects on CVL viscosity and pH (Table 3, figure 3), but had no impact on CVL density or osmolality. As expected, the median pH of CVL was highest among women with BV (pH 4.9), 4.6 among those with intermediate flora, and 4.5 among those having a *Lactobacillus*-predominant flora ($p < 0.001$). As shown in figure 3, there was an inverse linear relationship between CVL viscosity and pH, such that women with BV also had significantly decreased CVL viscosity ($p = 0.008$). In contrast to the impact of DMPA and menopausal status on CVL protein content, women with bacterial vaginosis had similar protein content in the CVL as compared to women with normal vaginal flora (table 3).

Comment

Hormonal contraceptive use was associated with increased viscosity of the CVL, but only in the presence of *Lactobacillus*-predominant vaginal microflora. Bacterial vaginosis was associated with the highest pH and lowest viscosity of the CVL when compared to women with intermediate flora and *Lactobacillus*-predominant vaginal flora. The present study shows that bacterial vaginosis mitigates the increased viscosity of CVL associated with hormonal contraceptive use, suggesting that the presence of mucin-degrading enzymes characteristic of BV has a more significant impact on the viscosity of the cervicovaginal fluid than the presence of reproductive hormones. This finding could provide one biological mechanism by which BV increases risk of HIV-1 acquisition and transmission. The decreased viscosity of the CVL associated with BV could render the mucin-gel layer more permissive to pathogens.

Depot medroxyprogesterone acetate users had decreased protein content in the CVL when compared to LNG-IUD users; they were similar to postmenopausal women with respect to protein content, a finding consistent with the hypoestrogenic effect of DMPA. Decreased protein in CVL could indicate a decreased amount of mucus or a decreased quantity of antimicrobial proteins present in the cervicovaginal fluid. The changes in CVL protein content in women using DMPA and how these changes impact vaginal mucosal immune function deserves further study. Postmenopausal women also had decreased CVL protein content, which could account for the decreased CVL viscosity associated with

postmenopausal status. Bacterial vaginosis did not impact the protein content of CVL indicating that the decreased CVL viscosity seen in women with BV was not mediated by decreased CVL protein but was more likely due to mucin-degrading enzymes as discussed.

To our knowledge, this is the only study to objectively measure viscosity and determine protein content in a large number of women well characterized by hormonal status. We were able to recruit women who reported using three hormonal contraceptive methods with different types, doses, and administration routes of progestins, women in different phases of the menstrual cycle and postmenopausal women. Other proteomic studies of CVF have recruited small numbers of women and have not characterized women by hormonal status[28, 29]. As shown in the present study, women using different hormonal contraceptives have different amounts of CVL protein, thus further proteomic studies of CVF should characterize and control for contraceptive use, menopausal status and phase of menstrual cycle.

Due to the small sample size among the groups, we were unable to adjust for the demographic characteristics that were different between hormonal groups. In the present study, large standard deviation values indicate significant variation in the physical and biochemical characteristics of CVF between women, making differences more difficult to detect. In addition, classification of hormonal status was determined by participant report and was not verified by measurement of reproductive hormones in plasma. Despite these limitations, we had sufficient power to detect some differences that warrant further study. Using a paired analysis by collecting samples from the same woman before and after contraceptive initiation and in different phases of her menstrual cycle should be considered to control for the significant population-level variation.

Although both CVF and CVL samples were collected in this study, direct measurements of the physical properties was possible only for the CVL samples. The CVF sample had a very small per participant volume of approximately 200 microliters. Thus, the absolute volume available for these assays was limited, and during the performance of the assays a significant proportion of the sample volume was lost because of its adherence to glassware. In addition, the viscosity of these samples was of such a high magnitude that it exceeded the capacity of the viscometer to reproducibly provide an accurate measurement of viscosity. By collecting the cervicovaginal fluid by introducing sterile normal saline into the vagina and performing a lavage, adequate sample volumes are generated for a broad range of assays.

In conclusion, our study indicates that when compared to reproductive hormones, bacterial vaginosis has a more significant impact on the physical properties of cervicovaginal lavage. In addition, women using DMPA for contraception have decreased CVL total protein content when compared to other premenopausal women, especially users of the LNG-IUD. Further studies should explore what are the differences in specific protein components of vaginal fluid and why women using DMPA have less cervicovaginal protein compared with other hormonal contraceptive users.

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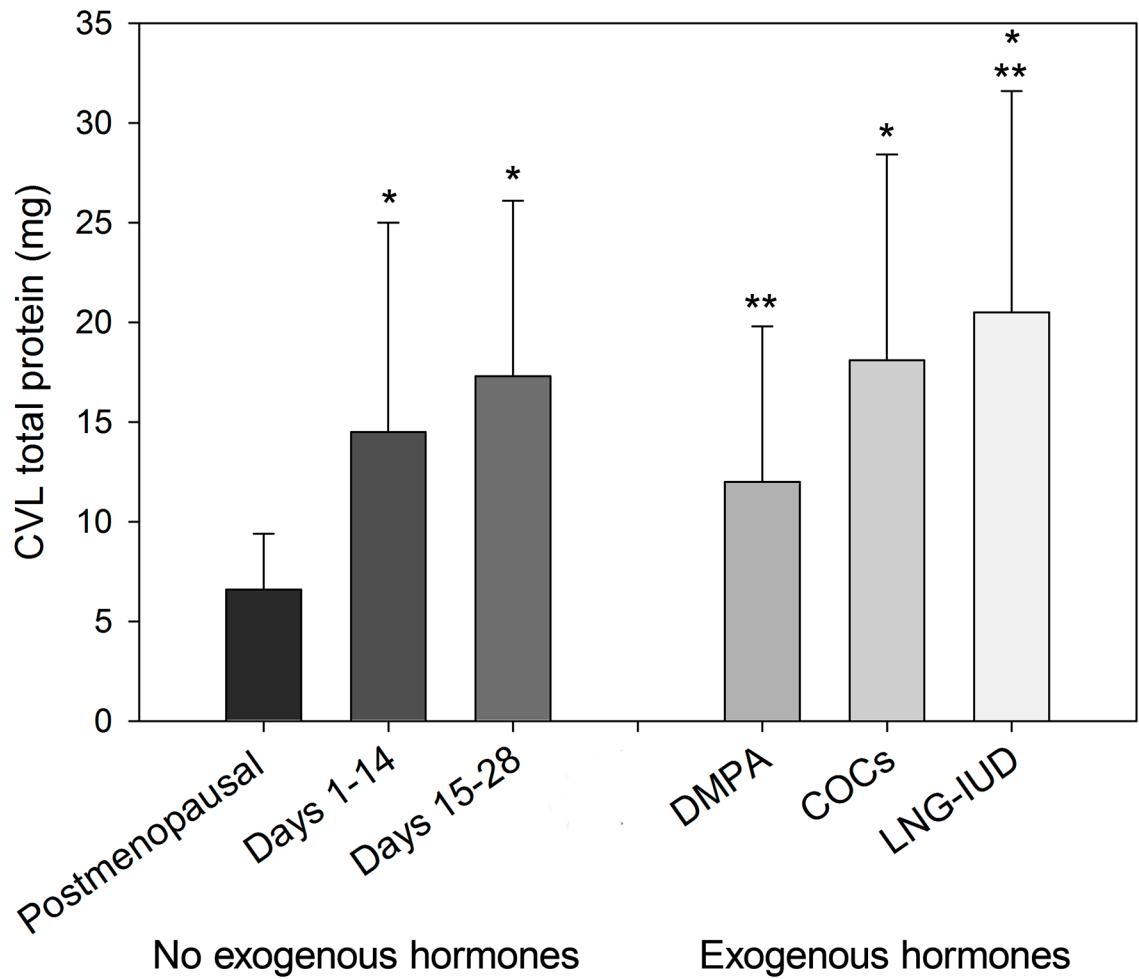


Figure 1. The Effect of Reproductive Hormones on Total Protein Content of CVL (mg)

* P < 0.05 Premenopausal group vs. Postmenopausal

** P = 0.008 DMPA vs. LNG-IUD

Figure one shows a bar graph of the mean CVL total protein content (mg) for each group. Postmenopausal women had less protein than premenopausal women, with the exception of women using DMPA.

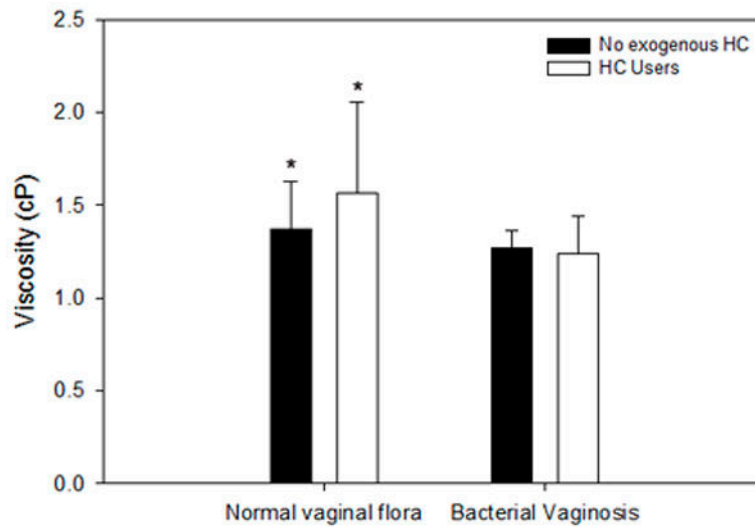


Figure 2. The Effects of Hormonal Contraceptive (HC) Use on the Viscosity of CVL (cP)

* P-value = 0.02 for hormonal contraceptive users vs. premenopausal women not using hormonal contraceptives with normal vaginal flora

Figure 2 compares the mean viscosity for premenopausal women using hormonal contraception and those not using hormonal contraception. The presence of bacterial vaginosis mitigated the impact of hormonal contraception on cervicovaginal fluid viscosity.

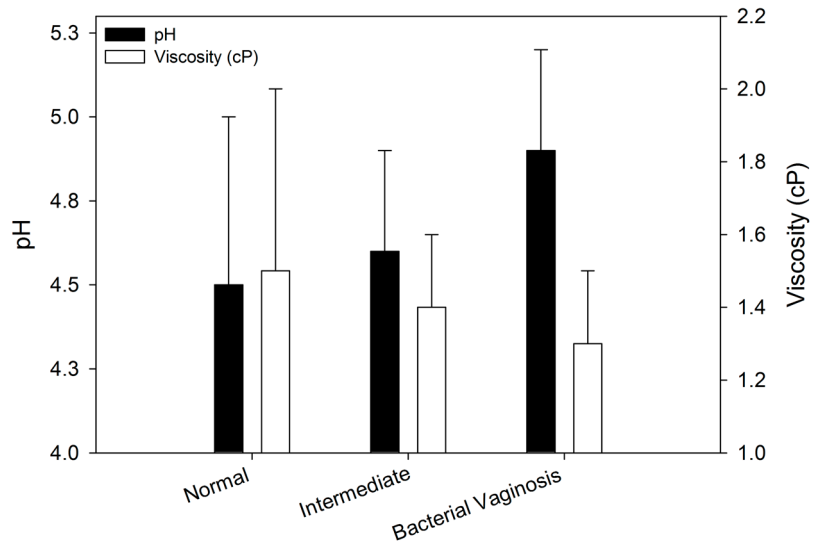


Figure 3. Effect of Vaginal Flora on the Physical Properties of CVL

Figure 3 shows the effect of vaginal microflora (determined by Nugent score) on viscosity and pH of CVL.

Table 1

Demographic Characteristics

	Days 1–14 (N=26)	Days 15–28 (N=27)	OCPs (N=27)	DMPA (N=28)	LNG-IUD (N=28)	Post-menopausal (N=29)
Age (mean ± SD)	29.8 ± 7.8	27.0 ± 6.5	28.6 ± 9.5	29.4 ± 6.1	29.0 ± 5.2	56.2 ± 7.0
BMI (mean ± SD)	30.8 ± 9.8	25.1 ± 6.5	25.4 ± 5.9	27.3 ± 6.4	27.6 ± 6.2	30.6 ± 7.0
Race						
White	14 (53.8%)	14 (51.9%)	24 (88.9%)	9 (32.1%)	22 (78.6%)	22 (75.9%)
Black	10 (38.5%)	7 (25.9%)	2 (7.4%)	19 (67.9%)	5 (17.9%)	7 (24.1%)
Asian	0	3 (11.1%)	1 (3.7%)	0	1 (3.6%)	0
Other	2 (7.7%)	3 (11.1%)	0	0	0	0
Condom use (most or all of the time)	10 (38.5%)	13 (48.1%)	3 (11.1%)	6 (21.4%)	4 (14.3%)	0
Current # partners						
None	12 (46.2%)	5 (18.5%)	7 (25.9%)	7 (25.0%)	4 (14.3%)	14 (48.3%)
One	12 (46.2%)	21 (77.8%)	19 (70.4%)	20 (71.4%)	22 (78.6%)	15 (51.7%)
Two or more	2 (7.7%)	1 (3.7%)	1 (3.7%)	1 (3.6%)	2 (7.1%)	0
Current smoker	6 (23.1%)	5 (18.5%)	3 (11.1%)	11 (39.3%)	2 (7.1%)	4 (13.8%)
Bacterial Vaginosis	8 (30.7%)	5 (18.5%)	1 (3.7%)	8 (28.6%)	2 (2.1%)	N/A

Table 2
 Biochemical and Physical Properties of Cervicovaginal Lavage by Hormonal Group

	Density (g/mL)	Osmolality (Osm/kg)	pH	Viscosity (cP)	Total Protein (mg)
Postmenopausal	1.0 ± 0.01	431.1 ± 12.3	5.7 ± 0.9	1.1 ± 0.2	6.6 ± 2.8
Premenopausal (Days 1–14)	1.0 ± 0.01	432.3 ± 11.5	4.7 ± 0.4	1.3 ± 0.2	14.5 ± 10.5
Premenopausal (Days 15–28)	1.0 ± 0.01	434.5 ± 10.0	4.7 ± 0.8	1.4 ± 0.3	17.3 ± 8.8
Combined Oral Contraceptives	1.0 ± 0.01	434.3 ± 11.3	4.5 ± 0.5	1.5 ± 0.4	17.8 ± 10.3
DMPA	1.0 ± 0.01	431.7 ± 8.7	4.5 ± 0.3	1.4 ± 0.2	12.0 ± 7.8
LNG-IUD	1.0 ± 0.01	431.6 ± 13.9	4.5 ± 0.4	1.6 ± 0.6	20.5 ± 11.1
P-Value*	0.1	0.8	<0.001 [†]	<0.001	<0.001

Data presented as mean ± standard deviation

* P-value from one way analysis of variance

[†] P-value = 0.1 among premenopausal groups

Table 3
 Biochemical and Physical Properties of Cervicovaginal Lavage by Vaginal Flora (Excluding postmenopausal women)

	Density (g/mL)	Osmolality (Osm/kg)	pH	Viscosity (cP)	Total Protein (mg)
Normal (n=90)	1.0 ± 0.01	433.0 ± 12.0	4.5 ± 0.5	1.5 ± 0.5	16.5 ± 8.9
Intermediate (n=23)	1.0 ± 0.01	434.3 ± 9.3	4.6 ± 0.3	1.4 ± 0.2	19.0 ± 12.9
Bacterial Vaginosis (n=23)	1.0 ± 0.01	431.0 ± 9.2	4.9 ± 0.3	1.3 ± 0.2	12.9 ± 9.0
P-Value*	0.3	0.6	<0.001	0.008	0.09

Data presented as mean ± standard deviation

* P-value from one way analysis of variance