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Original research article

A phase I randomized safety study of a single-size silicone rubber diaphragm used with or without a lactic-acid-containing diaphragm gel ☆,☆☆

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ABSTRACT

Objectives: To evaluate a lactic-acid-containing diaphragm gel (Contragel®) approved outside the United States for use with a silicone rubber diaphragm (Caya®). The study gel is being evaluated as a safer alternative to nonoxynol-9 (N-9) gel, which has been associated with risk of increasing susceptibility to human immunodeficiency virus (HIV).
Study design: This was a Phase I randomized, parallel study evaluating the safety of the novel diaphragm gel versus hydroxyethylcellulose (HEC) universal placebo gel delivered by the study diaphragm for two 7-day test cycles of daily use, without and with intercourse. The primary clinical safety endpoint was treatment emergent adverse events. Mucosal safety endpoints included colposcopic findings, anti-*Escherichia coli* activity of endocervical and vaginal fluid, immune mediators, Nugent score and ectocervical immune cell density. Endpoints were assessed prior to each test cycle and at day 7 of each test cycle. We compared the two independent groups and also evaluated paired changes from baseline in each gel cohort.
Results: Twenty-three participants used the study diaphragm with the novel gel (n=11) or with HEC (n=12). Use of either gel resulted in few genital AEs and no colposcopic findings. There were no differences in ectocervical histology and lymphocyte density or phenotype between the two cohorts at baseline or after each test cycle. We found no clinically important differences in the anti-microbial (anti *Escherichia coli*) activity of endocervical or vaginal fluid or concentrations of genital immune mediators (e.g. anti-inflammatory secretory leukocyte protease inhibitor (SLPI) or pro-inflammatory mediator RANTES) between the two gel cohorts at any visit. There were no important paired changes from baseline among participants using either gel in Nugent score, ectocervical histology or anti-microbial activity of genital secretions.
Conclusions: We found no clinically significant differences in clinical and mucosal safety endpoints between the two cohorts. The mucosal safety profiles of the study gel and HEC placebo gel were similar.
Implications: Our data demonstrate no clinically important differences between the safety profiles of the lactic-acid-containing diaphragm gel versus HEC placebo gel when used with the study diaphragm. N-9 can no longer be used with contraceptive diaphragms in high HIV prevalence regions. Although larger studies are needed, the novel gel appears safe for use with the study diaphragm, which is the first over-the-counter, non-hormonal, diaphragm.

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1. Introduction

Barrier contraceptives are an important alternative for women who do not use hormonal contraceptives or who prefer an event driven method. PATH and CONRAD developed the single-size, reusable, silicone rubber diaphragm, formerly known as SILCS [1–3] and now branded as the Caya® diaphragm. The pivotal contraceptive study was a multi-center trial in 450 couples randomized to use this diaphragm with BufferGel® (n=300) or 2% nonoxynol-9 (N-9) (n=150) [4]. Unlike N-9, the lactic-acid-containing diaphragm gel ContraGel® has not been tested as a stand-alone spermicide in women. However, N-9 can no longer be used in regions with high human immunodeficiency virus (HIV) prevalence and incidence rates, due to its potential to increase the risk of HIV acquisition with frequent use [5]. The alternative, BufferGel®, has not been marketed due to lack of effectiveness as a multipurpose prevention product against pregnancy and HIV [6]. Therefore, the lactic-acid-containing vaginal gel Contragel®, a vaginal gel approved for use with barrier devices in Europe and other countries but not approved by the United States (US) Food and Drug Administration (FDA), is being evaluated as a gel for use with the single-size, non-latex diaphragm. In an earlier study, this diaphragm used with N-9 or with the novel gel reduced the average number of progressively motile sperm/high power field from 22.5 to 0 in a recent post coital study [3]. The primary objective of the present study was to assess safety (treatment emergent adverse events (AEs)) and mucosal endpoints associated with mucosal susceptibility to HIV) of the novel gel versus hydroxyethylcellulose (HEC) universal placebo gel, each delivered by the study diaphragm during two 7-day periods of daily use, with and without intercourse. We chose to assess colposcopy [7], lower genital tract histology [8,9], microbiota [10,11], select secreted immune mediators and genital tract lymphocytes [12], as these endpoints are known to be significantly altered with N-9 use, HIV activation invitro [13] and or HIV seroconversion [14–16]. We selected universal HEC placebo gel as the non-inflammatory control gel. It is a water-based gel formulation designed for use as an inert comparator in clinical trials of candidate vaginal microbicides for the prevention of sexually transmitted infections (STIs) and HIV [17] and has been used in several large clinical trials of anti-HIV microbicides as the placebo [18–21] without evidence of increased AEs nor increase in HIV incidence. A secondary objective was to assess paired changes from baseline in each gel cohort.

2. Materials and methods

2.1. Study design

This was a Phase I randomized, parallel, non-significant risk study of the lactic-acid-containing diaphragm gel versus HEC universal placebo gel delivered by the Caya diaphragm performed at the CONRADIntramural Clinical Research Center at Eastern Virginia Medical School (EVMS), in Norfolk, Virginia. The study Chesapeake Institutional Review board approved the study, which we registered with ClinicalTrials.gov(NCT02462954). Healthy, sexually active women aged 18–50 years old, who were not pregnant and not at risk for pregnancy due to tubal sterilization and at low risk for HIV or STIs in a mutually monogamous relationship were eligible. They were ineligible if they were breastfeeding, within 2 months of last pregnancy outcome, using hormonal contraceptives or an intrauterine device, or had an unevaluated abnormal Pap test, genital findings suspicious for an STI, current v2aginal infection, or a urinary tract infection. We assessed vaginal secretions and cells for bacterial vaginosis (BV) using a Nugent score [22] and detected yeast vaginitis using light microscopy of genital secretions. Volunteers with a Nugent score of 7 or higher at screening (visit 1, V1) were not eligible to continue and the development of symptomatic BV after screening was a cause for discontinuation. Male partners had to be ≥18 years of age, at low risk for STIs, and have no known history of allergy or sensitivity to study products.

2.2. Randomization

We randomized participants in a 1:1 ratio to the study gel or HEC placebo gel at visit 3 (V3). The study biostatistician (TC) communicated treatment assignments via sealed envelopes with the randomization number on the outside and treatment code on the inside. No allocation errors occurred.

2.3. Study products

The study diaphragm (Caya, manufactured by Kessel medintim GmbH, Germany) is made of silicone rubber molded over a circular nylon spring that allows it to fit a range of women. The study gel (ContraGel, manufactured by DeltaMed GmbH, Germany) is designed for use with cervical barriers. It contains water, lactic acid, sodium lactate, cellulose and sorbic acid as active ingredients [3]. HEC universal placebo gel (manufactured by DPT Laboratory, Ltd., San Antonio, TX, USA) contains HEC as the gel thickener, purified water, sodium chloride, sorbic acid and sodium hydroxide.

2.4. Study assessments

We saw each participant for 6 visits over approximately 3 months, with approximately 2 weeks between each cycle, as outlined in Table1. Each participant used the study diaphragm with their assigned gel for 7 consecutive nights, leaving the diaphragm in place for 6–12 h each night for two cycles. The test cycles started during the early luteal phase of the menstrual cycle (based on reported menstrual calendar day) and completed in the late luteal phase, prior to expected menstruation. This allowed for approximately 14 days between test cycles. In test cycle one, no vaginal intercourse was permitted. During test cycle 2, we instructed participants to have two acts of vaginal intercourse (without condoms or additional lubricants) with the study diaphragm in place, with the second sex act taking place 6–12 h before the final study visit (visit 6, V6).

We collected information on AE's at each visit and graded these as related or unrelated to study product use or study procedures. We also asked participants, at each visit, if their male partners had reported any urogenital AEs. AEs were graded according to the National Institutes of Health (NIH) DAIDS table for grading the severity of AEs (https://rsc.niaid.nih.gov/clinical-research-sites/daids-adverse-event-grading-tables). We obtained one ectocervical tissue biopsy with a 3mm X 5mm Tischler forcep at baseline (visit 2, V2) and after test cycle 1 (visit 4, V4) and test cycle 2 (visit 6, V6) to characterize histology (epithelial thickness, number of epithelial cell layers), immune cell density and phenotype. We performed a colposcopic exam prior to and after test cycles 1 and 2at V3, visit 5 (V5) and V4, V6 respectively. Using FLOC tipped Copan swabs (Regular Copan FLOQ with 80mm breakpoint distance supplied in a sterile dry container: catalog # 552C for vaginal secretions and catalog # 53080C for endocervical secretions), we obtained endocervical and vaginal fluid and secretions for study endpoints outlined in Table1 prior to and after each test cycle.

We collected vaginal fluid and epithelial cells for gram stain and Nugent scoring [22] and sent another swab containing vaginal secretions on ice to the University of Pittsburgh for semi-quantitativeconcentrations of vaginal bacterial species (Appendix 1).

Table 1
Summary of CONRAD 127 study visits.

	Baseline Cycle		Test Cycle 1: 7 days of use without Intercourse		Test Cycle 2: 7 days of use with 2 acts of vaginal intercourse	
Visits	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
Menstrual cycle phase	Any	Luteal	Early luteal	Late luteal	Early luteal	Late luteal
	Screening	Baseline	Pre Test Cycle 1	Post Test Cycle 1	Pre Test Cycle 2	Post Test Cycle 2
Informed consent/partner information and screening labs (pregnancy, STI, Pap and HIV testing) and Exam. Assess diaphragm fit.	✓					
Randomize to Gel (ContraGel or HEC)			✓			
Vaginal fluid for prostate specific antigen testing		✓	✓	✓	✓	✓
Vaginal fluid pH, Gram stain and semi-quantitative vaginal cultures			✓	✓	✓	✓
Colposcopy			✓	✓	✓	✓
Endocervical and vaginal fluid for soluble markers of inflammation			✓	✓	✓	✓
Endocervical and vaginal fluid for anti-microbial activity			✓	✓	✓	✓
Ectocervical biopsy for histology, immune cell activation and phenotype		✓		✓		✓

Participants kept a paper diary recording sexual intercourse and we confirmed the absence of semen in the vagina at visits 2–5 and the presence of semen in the vagina at V6 with a point-of-care prostate specific antigen (PSA) test (ABA Card, Abacus Diagnostics, West Hill, CA). The details of sample processing and evaluation methods are included in Appendix 1.

2.5. Statistical methods

The sample size was based on feasibility rather than being powered for a specific safety endpoint comparison, and therefore we cannot exclude a type I statistical error, except where p values were calculated with a Fisher exact test. The main objective was to compare the two gel cohorts. Because this was a longitudinal study, we were also able to compare changes from baseline in a paired manner. We used independent-samples *t* test for normally distributed data and a Wilcoxon–Mann-Whitney test for non-normally distributed data to compare endpoints between the HEC placebo gel versus the study gel cohorts. Fisher exact test was used for comparing categorical variables between the two cohorts. Despite randomization, we noted that the cohorts had clinically important differences in the concentrations of *Lactobacillus*species at baseline (V3) prior to product use in test cycle 1. Because the Nugent score is highly correlated with vaginal bacterial species and vaginal pH [22], we fit ANCOVA models where Nugent score was the dependent variable and vaginal pH and semi-quantitative vaginal flora were covariates [22] to calculate an adjusted p value to account for these differences.

To assess paired changes between baseline and follow-up visits per gel group, repeated measured ANOVA was used in order to take into account the within and between time variability. Statistical significance was determined at α=0.05.

3. Results

3.1. Disposition of patients and baseline demographics

We screened 32 women; 23 women enrolled and completed baseline sampling and at least one of the 7-day treatment periods and 20 completed all visits (Fig.1). Eleven participants used the study gel, while 12 used HEC placebo gel. Participants in the gel groups had similar baseline demographic characteristics and prior experience with using a diaphragm (Table 2).

3.2. Comparison of gel cohorts

3.2.1. Safety

There were 27 AEs (HEC=21 and study gel=6) reported among 11 participants (HEC=7 and study gel=4) during the study (Fisher's exact p value=0.41). Only two participants reported AEs that were determined to be gel related. A HEC gel user developed vulvovaginal candidiasis (VVC) and vulvovaginal pruritus of moderate severity with intercourse and a study gel user reported vaginal burning with gel insertion of mild severity without intercourse. One report of symptomatic BV and one of VVC in the study gel group were not to be gel-related. No clinically relevant pelvic examination or abnormal colposcopic findings were observed in either gel group (data not shown).

3.2.2. Semi-quantitative assessment of vaginal bacteria and Nugent score

Prior to test cycle 1, at V3, despite randomization, a higher proportion of participants randomized to using the study diaphragm with the study gel had 4+ growth of hydrogen peroxide producing (H_2O_2+) *Lactobacillus* species (7/11, 63.6%) versus participants randomized to using the diaphragm with HEC gel (2/12 16.7%) (exact p=.04). At baseline, participants randomized to using the diaphragm with HEC gel also had a higher proportion of participants with 4+ growth of total *Lactobacillus* (H_2O_2+ and H_2O_2-) species (7/12, 58.3%) versus women randomized to using the diaphragm with the study gel (1/11, 9.1%) (exact p=.01). The proportion of participants in the diaphragm with HEC cohort with 4+ growth of *Gardnerella vaginalis* (7/12, 58.3%) at V4 and 4+ growth of *Myoplasma hominus* (5/10, 50%) at V6 was greater than that among the diaphragm with study gel users (0% of participants with 4+ growth of these species at V4 and V6 respectively). No apparent differences emerged between the two study product cohorts, at any visit for the other bacterial species cultured.

3.2.3. Inhibition of E.coli by endocervical and vaginal fluid

No apparent differences emerged between the cohorts in the anti-microbial activity of endocervical or vaginal fluid, based on the raw data or after the comparisons were adjusted for differences in the Nugent score (Supplemental Table 1).

3.2.4. Ectocervical histology and immune cell density and phenotype

We found no notable differences in any histologic parameter (epithelial thickness or number of ectocervical epithelial cell

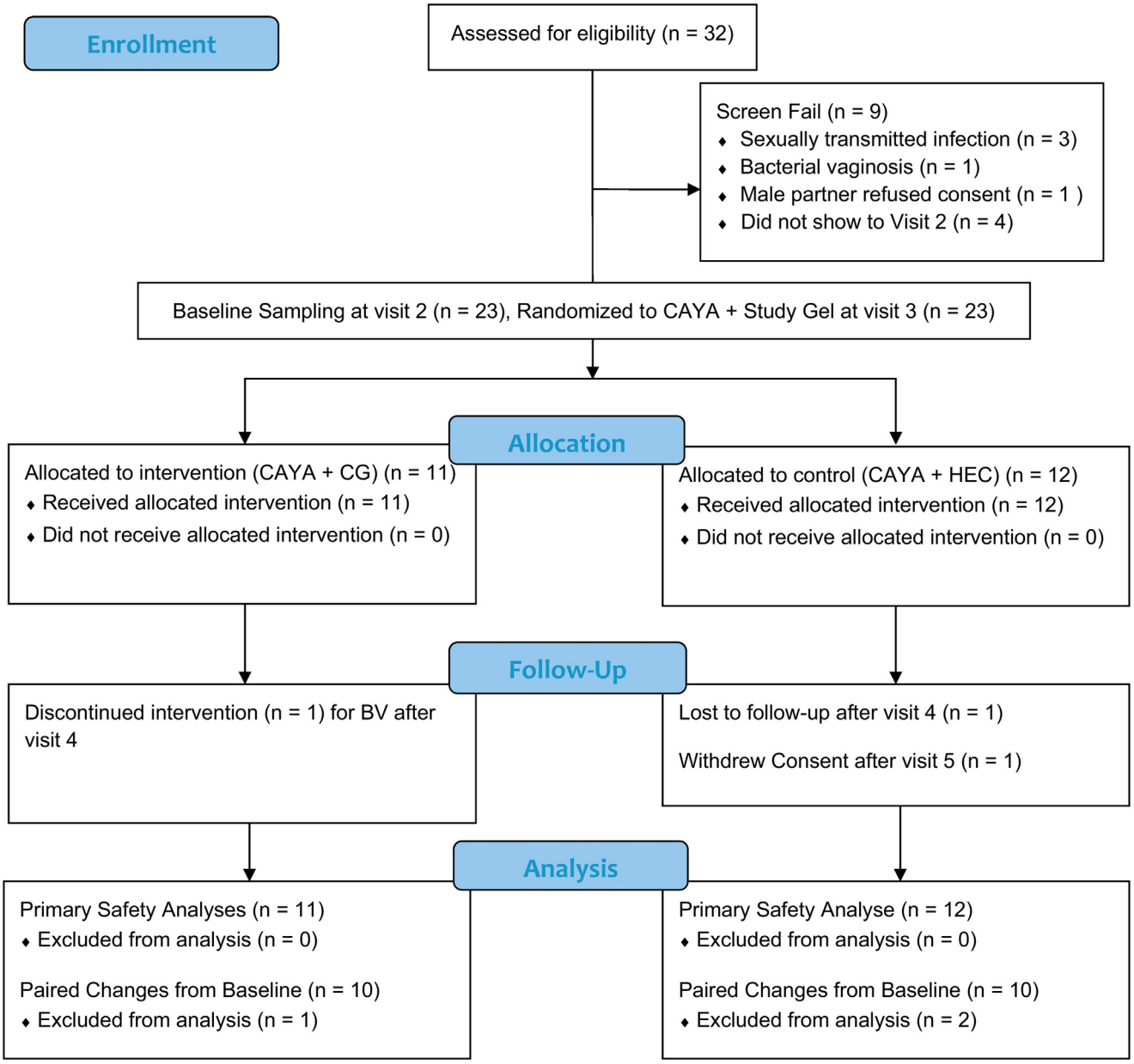

Fig. 1. CONRAD A13–127 CONSORT Flow diagram.

layers) (data not shown) between the gel cohorts. There were also no differences in the density or phenotype of ectocervical immune cells at any visit between the two cohorts. Selected immune cell population data collected at V2, V4 and V6 appear in Table 3 and Supplemental Table 1.

3.2.5. Immune mediators in the endocervical and vaginal secretions

After adjusting for Nugent score at each visit, we found no clinically meaningful differences in any endocervical or vaginal secreted immune mediator at any visit (Table 3, Supplemental Table1). Immune mediators not shown in Table 3 or Supplemental Table1 had no clinically meaningful differences between the groups. Chemokine (C-C motif) ligand 5 (CCL5), also known as RANTES (regulated on activation, normal T cell expressed and secreted) and secretory leukocyte protease inhibitor (SLPI) are displayed for each visit in Supplemental Table1 based on their relevance to HIV acquisition, demonstrated in previous longitudinal cohorts [14–16].

3.3. Comparison of changes from baseline in HEC and GC gel users

Tables 4a (for the diaphragm with HEC gel cohort) and 4b (for the diaphragm with study gel cohort) show endpoints with potential relevance to mucosal safety. For all assessed endpoints, we found no clinically meaningful changes from V3 to V5 (the start of both test cycles), demonstrating that baseline, pre-test cycle values were consistent throughout this longitudinal study (data not shown). Changes in endpoints between post product use visits (V4 and V6), showing the effect of sexual intercourse, were not part of the planned analysis and were deemed to be beyond the scope of this manuscript and are thus not reported.

Table 2
Baseline demographic characteristics of women randomized to the study gel versus Hydroxyethylcellulose placebo gel.

Variable	Study gel* (*n*=11)N (%) or Mean (SD)	Hydroxyethylcellulose gel (*n*=12)N (%) or Mean (SD)	p Value
Age (y)	37.5 (6.2)	37.6 (6.4)	.98
Years of education	14.5 (2.2)	16.1 (3.0)	.27
Weight (lb)	179.4 (46.7)	188.5 (36.7)	.41
Height (inches)	63.7 (3.0)	64.9 (2.1)	.34
Ethnicity			1.00
Hispanic	0 (0.0)	1 (8.3)	
Not Hispanic	11 (100.0)	11 (91.7)	
Race			.23
Black or African American	7 (63.6)	4 (33.3)	
More than one race	0 (0.0)	2 (16.7)	
White	4 (36.4)	6 (50.0)	
Study partner status			1.00
Living with study partner	9 (81.8)	9 (75.0)	
Not living with study partner	2 (18.2)	3 (25.0)	
Diaphragm use in the past			.90
No	8 (72.7)	9 (75.0)	
Yes	3 (27.3)	3 (25.0)	
Pregnancy history			
Mean gravidity	1.5 (1.4)	1.9 (2.2)	.80
Mean parity	1.1 (1.3)	1.3 (1.4)	.87

* The study gel was a lactic-acid-containing diaphragm gel, Contragel.

3.4. Semi-quantitative assessment of vaginal bacteria and Nugent score

Cell size was too small to apply statistics to comparison of semi-quantitative vaginal flora concentrations in paired comparisons; however, there were no clinically meaningful changes in Nugent score with use of the study diaphragm and either gel, with changes from baseline with either gel, in either test cycle ranging from−0.2 to−1.2 (Tables 4a and 4b).

3.4.1. Inhibition of E.coli by endocervical and vaginal fluid

When vaginal and endocervical fluid anti-*E.coli* activity data were pooled or analyzed separately, there were no notable changes from baseline in either test cycle of the anti-*E.coli* activity of the secretions among HEC or study gel users (Tables 4a and 4b).

3.4.2. Ectocervical histology and immune cell density and phenotype

Diaphragm with HEC users had increases in lamina propria (LP) immune cells (CD45+ and CD3+) (Table 4a), while there were no changes in any epithelial (EPI) or LP immune cell with diaphragm with study gel use (Table 4b). We found no changes in ectocervical histology (epithelial thickness, number of epithelial cell layers) when the study diaphragm was used with either gel, in either test cycle (data not shown).

3.4.3. Immune mediators in the endocervical and vaginal secretions

There were changes from baseline in secreted mediators when the diaphragm was used with either gel, with generally more substantial changes in the vaginal fluid compared to changes in endocervical fluid and more changes in the diaphragm with HEC than the diaphragm with study gel cohorts (Tables4a and 4b).

4. Discussion

N-9-containing spermicides have typically been used in conjunction with diaphragms, but the surfactant properties of N-9 cause epithelial disruption, alteration of vaginal microbiota and inflammation, particularly among frequent users [7–13] (reviewed in [23]), and have been reported to increase the risk of HIV infection [5]. Gel alternatives to N-9 to be used with the diaphragm are greatly needed, but no published safety data are available. This study found no clinically important differences in AEs, colposcopic findings and mucosal safety biomarkers among participants who used the study diaphragm with the study gel versus those who used the diaphragm with HEC placebo gel.

Table 3
Significant independent group comparisons between the study diaphragm* with the study gel** versus the study diaphragm with HEC placebo gel.

Visit	HEC			STUDY GEL			p Value	Adj p value
	N	Mean	SD	N	Mean	SD		
Nugent Scores								
Post Test Cycle 1 (no sex)	12	5.4	3.3	11	2	2.7	.02	
Pre Test Cycle 2 (with sex)	10	5.4	4.3	10	2.8	3.0	.03	
Vaginal pH								
Post Test Cycle 1 (no sex)	12	5.1	1	11	4.1	0.2	.01	
Selected ectocervical immune cells (cells/mm^2) in the epithelium (EPI) or lamina propria (LP)								
Post Test Cycle 1 (no sex)								
CD4_EPI	12	0.1	0.5	11	1.2	1.6	.04	.10
Endocervical fluid								
Pre Test Cycle 1 (no sex) selected immune mediators (pg/mg total protein)								
SLPI (X 10^6)	12	2.7	3.4	11	1.5	1.3	.01	.16
Post Test Cycle 1 (no sex) selected immune mediators (pg/mg total protein)								
GM-CSF	12	2.5	2.3	11	0.9	0.3	<.01	.59
Vaginal fluid								
Pre Test Cycle 1 (no sex) selected immune mediators (pg/mg total protein)								
GMCSF	12	1.5	1.5	11	0.8	0.8	.01	.56
Post Test Cycle 1 (no sex) selected immune mediators (pg/mg total protein)								
GM-CSF	12	3.5	3	11	1.6	1.2	<.01	1.00
IL10	12	2.2	1.5	11	1	0.8	.04	.55
IL1a	12	1216	1154	11	382	415	.04	.98
Pre Test Cycle 2 (with sex) selected immune mediators (pg/mg total protein)								
GM-CSF	10	2.4	1.4	9	1.1	0.9	.04	.62
IL6	10	13.9	23.7	9	1.2	0.9	.02	.33

The study diaphragm was the single-size silicone rubber diaphragm, CAYA.
The study gel was the lactic-acid-containing diaphragm lubricant, Contragel.

Table 4a
Changes from baseline in selected mucosal endpoints among paired samples from study diaphragm* with HEC gel users. Bolded values are greater than comparator in each row (p value<.05).

Endpoint study diaphragm with HEC Gel	Product use without intercourse				Product use with intercourse			
	Difference in Test Cycle 1 (Post Cycle 1 Visit – Pre Cycle 1 Visit)				Difference in Test Cycle 2 (Post Cycle 2 Visit – Pre Cycle 2 Visit)			
	N	Mean difference	S.D.	p Value	N	Mean difference	SD	p Value
E. coli inhibition by endocervical or vaginal secretions (% inhibition)								
Endocervical	12	26	43.3	.08	10	30.4	42.6	.06
Vaginal	12	−8.3	28.4	.23	10	−20.3	35.8	.08
Microflora parameters								
Nugent Score	12	−0.5	0.9	.22	10	−0.7	2.1	.34
Vaginal pH	12	0.7	0.9	.07	**10**	**0.6**	**0.6**	**<.01**
Selected immune mediators (pg/mg total protein) in endocervical secretions								
IL-8	**12**	**4464.5**	**5445**	**.02**	10	3251.1	10,246.7	.85
IL-1a	12	115.7	1115.2	.79	**10**	**−691.9**	**1259.5**	**.03**
MIP-1a	**12**	**25.2**	**44**	**.04**	10	2.7	28.5	1.00
Selected immune mediators (pg/mg total protein) in vaginal secretions								
IL-8	**12**	**165.8**	**4421.2**	**.03**	10	−2472.2	9939.6	.43
GM-CSF	**12**	**2**	**2.5**	**<.01**	10	1.7	3.1	.19
IL-10	**12**	**1.4**	**1.6**	**<.01**	**10**	**2.3**	**3.5**	**.04**
MIP-1a	**12**	**78.5**	**128.9**	**.03**	10	32.7	102	.28
TNFa	**12**	**2.5**	**3.2**	**<.01**	10	3.8	5.1	.06
BD2	12	−47,858.8	77,816.9	.13	**10**	**−95,797.6**	**185,765.8**	**.01**
IL-1RA	12	−139,292.4	579,556.1	.52	**10**	**−557,329.6**	**1,004,352**	**.04**
Ectocervical immune cells (cells/mm^2)	Difference between Post Cycle 1 – Baseline				Difference between Post Cycle 2 – Baseline			
In lamina propria (LP) or epithelium (EPI)	N	Mean Difference	STD	p Value	N	Mean Difference	STD	p Value
CD45_LP	12	15.6	36.6	.15	**9**	**53.7**	**87.9**	**.02**
CD3_LP	**12**	**24.8**	**35.1**	**.05**	**9**	**49.6**	**73.1**	**.03**

Table 4b
Changes from baseline in selected mucosal endpoints among paired samples from study diaphragm with study gel* users. Bolded values are significantly greater than comparator in each row (p<.05).

Endpoint CAYA with Contragel	Product use without intercourse				Product use with intercourse			
	Difference in Test Cycle 1 (Post Cycle 1 Visit – Pre Cycle 1 Visit)				Difference in Test Cycle 2 (Post Cycle 2 Visit – Pre Cycle 2 Visit)			
	N	Mean Difference	S.D.	p Value	N	Mean difference	S.D.	p Value
E. coli inhibition by endocervical or vaginal secretions (% inhibition)								
Endocervical	11	21.3	44.4	0.10	10	7.7	14.7	0.16
Vaginal	11	6.3	38.8	0.72	10	−30.6	46	0.10
Microflora parameters								
Nugent score	11	−1.2	3.1	1.00	10	−0.2	2.1	1.00
Vaginal pH	11	−0.1	0.6	1.00	10	0.4	0.7	0.38
Selected immune mediators (pg/mg total protein) in endocervical secretions								
GM-CSF	**11**	**−0.5**	**0.5**	**0.01**	10	−0.9	2.3	0.43
RANTES	11	30	83.6	0.15	10	−6	24.5	0.85
SLPI	**11**	**−1,102,253.7**	**1,405,713.3**	**0.03**	10	−1,171,043.8	2,394,590.3	0.19
Selected immune mediators (pg/mg total protein) in vaginal secretions								
IL-10	11	0.3	0.9	0.32	**10**	**1.1**	**1.8**	**0.05**
IP-10	11	6.8	96.1	0.83	**10**	**233.3**	**308.9**	**0.05**
MIP-1a	**11**	**22.1**	**29.9**	**0.04**	10	27.9	107.6	0.43
RANTES	11	7.7	24.7	0.15	**10**	**2.2**	**1.7**	**< 0.01**
TNFa	11	0.5	1.2	0.15	**10**	**3.7**	**6.4**	**0.05**
BD2	**11**	**−76,317.9**	**117,325.6**	**< 0.01**	10	−23,745.2	314,717.1	0.08
Ectocervical immune cells (cells/mm^2)	Difference between Post Cycle 1 – Baseline				Difference between Post Cycle 2 – Baseline			
In lamina propria (LP) or epithelium (EPI)	N	Mean Difference	STD	p Value	N	Mean Difference	STD	p Value
CD45_EPI	11	23.9	45.6	0.12	10	19.4	68.9	0.38
CD3_EPI	11	20.6	37.6	0.10	10	14.7	54.9	0.38

The study diaphragm was the single-size silicone rubber diaphragm, CAYA.
The study gel was the lactic-acid-containing diaphragm lubricant, Contragel.

Despite randomization, we found clinically important differences in the proportion of women having vaginal lactobacilli species between the two cohorts at baseline. We used post-hoc analyses to control for differences in Nugent score between the two groups, as the vaginal microbiome may impact vaginal immune cells [24], immune mediators (reviewed in [25]). However, it is reassuring that neither HEC nor the study gel caused clinically meaningful shifts in the Nugent score over the two test cycles, as N-9 is known to alter the vaginal microbiota [10,11]. There were also no clinically important differences in ectocervical histology, lymphocyte density or phenotype of immune cells in the EPI or LP between the two cohorts. Given that repeated use of N-9 causes significant disruption of the genital epithelium and increases in subclinical inflammation of the vaginal mucosa [12],

these data support the safety of both gels. We did find an increase in CD3 and CD45+ cells in the LP from baseline with HEC use, while this was not observed among study gel users. Our group previously showed alterations in ectocervical immune cells with BV [24], and it is possible that the higher rates of asymptomatic BV and reduced *Lactobacillus* species observed in the HEC cohort, despite randomization, could account for some of these immune cell changes. Neither gel resulted in large changes in activated lymphocytes (HLADR+) or HIV target cells (CD4+, CCR5+).

Immune mediators secreted in the lower genital tract have been proposed as early safety biomarkers for topically applied vaginal products, particularly HIV microbicides [13–16,26–29]. Subset analyses from two large studies in Africa found that alterations of RANTES, SLPI, MIP-1α, MIP-1β and IP-10 were associated with an increased risk of HIV seroconversion compared to matched controls [14–16]. However, concentrations of several of these secreted immune mediators are highly variable, have no clinically defined normal ranges and are known to change with reproductive tract infections such as BV (reviewed in [25]). We do report changes from baseline in some endocervical and vaginal secreted proteins after participants used the study gel, including decreases in SLPI and increases in RANTES,IP-10 and MIP-1α. However, these changes with were not consistent throughout both test cycles and often were isolated to either the endocervical fluid or vaginal fluid, making them likely clinically insignificant. As with the independent group comparisons, we acknowledge that our study is underpowered to detect statistically significant changes from baseline. However, we are reassured that HEC placebo gel has been used by thousands of women in large phase III trials [18–21] without safety issues and the study gel evinced fewer changes than HEC in the present study.

In addition, increases in cervico-vaginal (CV) *E.coli* inhibitory activity has been associated with an *L.crispatus* or *L.jensenii* dominant microbiome and may be a marker of mucosal health [30–34], whereas among young women with BV or a more diverse anaerobic microbiome, an increase in baseline low *E.coli* inhibitory activity was associated with inflammation and with increased risk of HIV seroconversion, as illustrated in a substudy of CAPRISA 004 and HPTN 035 [35,36]. Thus the finding of no change in this activity further supports the safety profile of the study gel.

This study has several strengths including being the first phase I clinical trial to evaluate relevant clinical and mucosal safety endpoints of the study gel, when used with the study diaphragm in both the absence and presence of sexual intercourse. However, a limitation of this phase I study was that the primary endpoint was treatment emergent AEs and the sample size was not powered to detect statistically significant changes between the gel groups. We are reassured by the fact that only one participant in each gel cohort reported a product related AE and both were mild events with quick resolution. Despite proper randomization, we found clinically significant differences in the semi-quantitative concentrations of Lactobacillus species between the groups, prior to product use, and therefore we adjusted the independent group comparisons for Nugent score, a clinically valid marker of vaginal flora. We do acknowledge that the other mucosal safety endpoints are markers of subclinical inflammation, which do not have established clinical normal ranges. We cannot rule out a type I error due to small sample size. Given the relevance of many study endpoints to mucosal HIV-1 acquisition, larger studies are needed to confirm our initial findings.

Because these mucosal endpoints may be altered by reproductive tract infections (RTIs) or STIs [24,30,37], semen exposure [34,38,39]endogenous hormonal changes [40] and exposure to inflammatory vaginal products such as N-9 [13], we attempted to eliminate or control for these exogenous factors in this study. However, it makes comparison of our findings to other studies of different designs among different populations difficult.

Recent acceptability data indicate that the contraceptive diaphragm remains a desired method [41,42], particularly for women cannot use or chose to avoid hormonal regimens. The approval of an over-the-counter, single size, ergonomic, diaphragm eliminates the need for a pelvic examination to determine size and fitting, making it potentially more accessible, cost effective and desirable to women worldwide, including women living in HIV endemic regions [2,43–45]. The study gel (ContraGel®) used with the study diaphragm (Caya®) represents a safe alternative diaphragm lubricant. These products may fill an unmet need for woman-controlled, non-hormonal, event-driven contraception.

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