Safety Analysis of the Diaphragm in Combination with Lubricant or Acidifying Microbicide Gels: Effects on Markers of Inflammation and Innate Immunity in Cervicovaginal Fluid

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Objective

Diaphragms are being considered for use with vaginal microbicide gels to provide enhanced protection against sexually transmitted pathogens. The purpose of this study was to determine whether use of a diaphragm with microbicide or placebo gel causes cervicovaginal inflammation or perturbations in cervicovaginal immune defense.

Method of study

Eighty-one non-pregnant women were randomized into three groups and instructed to use Milex® (CooperSurgical, Inc., Trumbull, CT, USA) diaphragms overnight for 14 days in combination with one of the two acid-buffering microbicide gels [ACIDFORM™ (Instead Inc., La Jolla, CA, USA) or BufferGel™ (BG; ReProtect Inc., Baltimore, Maryland)] or placebo gel (K-Y Jelly®; Personal Products Inc., Raritan, NJ, USA). Cervicovaginal lavages (CVLs) were performed prior to study entry and on days 8 and 16. Nine soluble mediators of vaginal inflammation or immune defense were measured in CVLs by Bio-Plex or ELISA.

Results

Use of diaphragms with placebo or microbicide gel was not associated with increased levels of inflammation markers. Concentrations of secretory leukocyte protease inhibitor (SLPI) were markedly reduced in the BG group.

Conclusion

Daily use of a diaphragm with placebo or acidifying microbicide gel did not cause cervicovaginal inflammation. However, diaphragm/BG use was associated with markedly reduced levels of SLPI, an important mediator of innate immune defense. Further studies are warranted to establish the safety of diaphragm/microbicide gel combinations.

Introduction

The diaphragm is a widely accepted reusable method of female-controlled contraception that has a relatively low cost.¹ Although male condom use has the highest documented efficacy for the prevention of sexually transmitted infections (STIs), including HIV-1,² many women are unable to negotiate condom use with their partners. Diaphragms, which cover the cervix, may be effective in reducing the transmission of STI pathogens that primarily target the cervix, such as *Chlamydia trachomatis*, *Neisseria gonorrhea* and Human papillomavirus.³ In addition, because HIV-1 target cells are concentrated in the cervical transformation zone,^{4,5} and evidence suggests that this is a site of early infection,⁶ it has been hypothesized that diaphragms may also reduce the sexual transmission of HIV-1. A recent large randomized controlled clinical trial of diaphragm with lubricant gel use for prevention of HIV acquisition in African women failed to show a protective effect against HIV infection, but the study also reported lower condom use in women provided with diaphragms.⁷

Topical microbicides are being developed to prevent the sexual transmission of HIV and other pathogens, but several leading microbicide candidates [Nonoxynol-9 (N-9), PRO-2000, cellulose sulfate, Savvy and Carraguard®] have failed in Phase III clinical efficacy trials.⁸ Failure has been attributed to: (i) limited potency *in vivo*, (ii) inconsistent usage, and (iii) HIV potentiating effects. Theoretically, use of safe microbicide gels in combination with diaphragms could be highly effective, as the diaphragm would serve both as a physical barrier to the cervix and to concentrate the gel around the cervix. Even if applied to the dome of the diaphragm, some of the gel will likely leak out over time, providing sustained release of gel into the vaginal cavity. Early investigations indicated that N-9 used in combination with a diaphragm, reduced infection rates of *N. gonorrhea*, *C. trachomatis* and *Trichomonas vaginalis*.³ However, N-9 use has been associated with vaginal lesions⁹ and a dramatic elevation of proinflammatory cytokines and inflammatory cells in cervicovaginal secretions,¹⁰ and may promote the sexual transmission of HIV-1.¹¹ Furthermore, N-9 use was associated with a reduction in levels of secretory leukocyte protease inhibitor (SLPI), an important mediator of vaginal immune defense.¹⁰ Current recommendations include the N-9/diaphragm combination as a

valuable contraceptive method but do not include its use to prevent HIV-1 transmission.¹¹

Two new acid-buffering vaginal microbicide candidates provide a potential alternative to N-9 for use with the diaphragm to reduce transmission of HIV-1 and other STIs. ACIDFORM™ (AF; Instead Inc., La Jolla, CA, USA) and BufferGel™ (BG; ReProtect Inc., Baltimore, MD, USA) exert potent microbicidal effects against a variety of bacterial and viral STI pathogens *in vitro* and *in vivo*,^{12,13} and have not caused vaginal irritation or inflammation in Phase I clinical trials.^{14,15} To assess this potential, we performed a randomized safety trial using a diaphragm with placebo gel [K-Y Jelly® (K-Y; Personal Products Inc., Raritan, NJ, USA)] or microbicide gel (BG or AF) and measured signs of cervicovaginal irritation and inflammation before, during and after daily use of diaphragm + gel over a 2-week period. Clinical observations from this study have been reported elsewhere.¹⁶ In this report, we describe concentrations of proinflammatory cytokines, chemokines and other markers of cervicovaginal inflammation and immune defense in cervicovaginal secretions. This approach can detect subtle inflammatory processes that are not readily detectable by clinical examination,^{10,17,18} and has begun to be used in vaginal microbicide Phase I clinical trials.^{19–21}

Materials and methods

Eighty-one sexually active women between 18 and 48 years of age were enrolled in a randomized Phase I safety trial to assess the effects of repeated cervical and vaginal exposure to placebo or microbicide gel used with a diaphragm, and all 81 produced samples for the first time point (day 5 to day 7 of the menstrual cycle). Sixty-eight women completed the study; detailed information on subject enrollment, demographics and compliance is published elsewhere.¹⁶ The placebo gel, K-Y Jelly®, was purchased from Personal Products Inc., Raritan, New Jersey. Two microbicide gels were evaluated: ACIDFORM™ and BufferGel™. Participants were instructed to abstain from sex for the duration of the study and use a fitted Miletex Wide-Seal® Arcing diaphragm (CooperSurgical, Inc.) with 5 cc gel for 6–10 hrs on 14 consecutive nights.

The initial evaluation occurred on day 5 to day 7 of the menstrual cycle to avoid effects of menses on vaginal cytokine levels. Cervicovaginal lavages (CVLs) were conducted with 5 cc of normal saline

prior to diaphragm/gel use (baseline) on day-8 of product use (2–4 hrs after removal of diaphragm and gel), and on day-16 (24 hrs after removal of diaphragm and gel for the last time). Each CVL was supplemented with 500 μ L of 10 $\times$ phosphate-buffered saline, centrifuged to pellet cells, and supernatants were frozen at -70°C until thawed for Bio-Plex/ELISA assays.

Markers of inflammation assessed in this study included three proinflammatory cytokines [Interleukin (IL)-1 α and β , Tumor Necrosis Factor (TNF)- α], four chemokines [IL-8; Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES); Macrophage Inflammation Protein (MIP)-1 α and β], and granulocyte elastase. These markers have previously been detected in CVLs and represent a broad range of inflammatory processes. Elevated levels of proinflammatory cytokines and chemokines have been associated with vaginal infections or signs of clinical inflammation.^{10,22–25} We also measured levels of SLPI, an anti-inflammatory factor detectable in CVLs, that may regulate vaginal inflammation and inhibit HIV-1 infection.²⁶ IL-1 α , IL-1 β , TNF- α , IL-8, RANTES and MIP-1 α were measured by Bio-Plex (Upstate Cell Signaling Solutions, Charlottesville, VA, USA). MIP-1 β and SLPI were measured by ELISA kits from R&D Systems (Minneapolis, MN, USA). Granulocyte elastase was measured by an ELISA system from BioVendor (Brno, Czech Republic). Lower limits of detection were 1 pg/mL for the Bio-Plex assays, 4 pg/mL for the R & D MIP-1 β assay, 25 pg/mL for the R&D SLPI assay, and 3 ng/mL for the BioVendor granulocyte elastase assay.

To determine the effects of diaphragm with gel on vaginal inflammatory markers, two-factor [one within-factor (sampling day) and one between-factor (gel type)] analysis of variance (ANOVA) was performed on the data. Following a significant ANOVA, pair-wise comparisons of the various groups were performed with Scheffé's *F post hoc* tests. The dependent variables (i.e., inflammatory and immunologic markers) in this study did not satisfy the conditions of normal distribution or homogeneity of variance. Therefore, logarithmic transformation of the data was undertaken to allow use of ANOVA. Statistical analysis was performed using StatView (version 5.0.1; SAS Institute Inc., Cary, NC, USA) and Prism (version 2.0b; GraphPad Software Inc., San Diego, CA, USA) statistical software. In all cases, statistical significance was assumed when $P < 0.05$.

Results

Inhibitory Effects of Microbicide Gels and K-Y Jelly® in ELISA and Bio-Plex Assays

To investigate the possibility that the microbicide or placebo gels interfere with the Bio-Plex/ELISA assays, we ran the standard curve for each assay under various pH conditions, and in the presence of 10-fold dilutions of gel, diluted in 1X PBS, pH 7.0, to replicate conditions of the samples. Addition of PBS alone in the pH 4–8 range to assay standards had little to no effect on the measurements (all values were >80% of untreated controls), whereas PBS at pH <4.0 inhibited many assays by >50% (data not shown). The pH of K-Y diluted in PBS was 7.0 for concentrations $\leq 10\%$; K-Y at concentrations $\leq 10\%$ in PBS did not inhibit any of the Bio-Plex or ELISA assays. The pH of AF and BG was <4.0 at concentrations >10%; 4.0 and 4.8, respectively, at a 10% concentration; and >5.0 at concentrations <5% in PBS. At a 10% concentration in PBS, AF and BG inhibited all assays by >50%; at a 1% concentration they were no longer inhibitory except for having a moderate effect in the chemokine assays (Fig. 1).

Effects of Diaphragm With Gel on Vaginal Inflammatory Markers in CVLs

Combined baseline (pre-treatment) values [median (25–75%)] for the 81 women across all three groups were as follows: IL-1 α [63.0 (27.9–178.5)pg/mL], IL-1 β [9.5 (2.2–45.7) pg/mL], TNF- α [2.8 (1.0–6.25) pg/mL], granulocyte elastase [93.5 (37.6–239.3) ng/mL], IL-8 [817 (258–1972) pg/mL], RANTES [38.0 (15.6–68.2)pg/mL], MIP-1 α [34.0 (0–78.6) pg/mL], MIP-1 β [0 (0–0) pg/mL], and SLPI [32.3 (19.6–34.6) ng/mL]. Levels of proinflammatory cytokines, chemokines, granulocyte elastase and SLPI did not differ between treatment groups at Baseline, nor were levels of any of the markers increased in Day-8 or Day-16 CVLs (when compared with Baseline) from any treatment group (Fig. 2). On the other hand, levels of some of the inflammatory markers and SLPI were decreased in Day-8 and Day-16 post-treatment CVLs. A significant main effect for sampling day indicated that levels of IL-1 α ($P < 0.01$), IL-1 β ($P < 0.01$) and granulocyte elastase ($P < 0.05$) were significantly lower in Day-8 CVLs when compared with Baseline (data pooled across treatment groups, Fig. 2). Furthermore, levels of

Fig. 1 Inhibition of ELISA and Bio-Plex assays by microbicide and placebo gels. Various concentrations of K-Y, AF and BG, diluted in PBS, were added to assay standards. Readings for standard curves obtained in the presence of gel were adjusted to percentage of normal standard curve (no gel).

IL-1 α were significantly lower in Day-8 CVLs when compared with Day-16 ($P < 0.05$), and levels of granulocyte elastase were significantly lower in Day-16 CVLs when compared with Baseline ($P < 0.05$). A significant interaction between gel type and sampling day indicated that levels of IL-8 were lowest in Day-8 CVLs from the BG treatment group (Fig. 2). Similarly, a significant interaction between gel type and sampling day indicated that concentrations of SLPI were significantly lower in both Day-8 ($P < 0.0001$) and Day-16 ($P < 0.01$) CVLs from the BG treatment group when compared with Baseline (Fig. 2). To expand this observation to include other markers of vaginal innate immunity, we conducted a sub-study in which we measured levels of lysozyme and lactoferrin by ELISA (R&D Systems) in Baseline and Day-16 samples from 12 women in the BG treatment group. A significant reduction in lysozyme was observed on Day-16 (medians 32.7 versus 14.5 ng/mL, $P = 0.0036$ by paired t -test) as when compared with baseline, but a significant effect on lactoferrin concentration was not observed (medians 854.0 versus 657.6 ng/mL, $P = 0.14$ by paired t -test).

Discussion

Assessment of cytokines and other immunologic markers in cervicovaginal secretions provides valuable information about lower genital tract inflammation and immune defense mechanisms.²⁷ Elevated

proinflammatory cytokine profiles have been associated with a variety of vaginal infections, including bacterial vaginosis,^{22,23} mycoplasma²⁴ and HIV-1.²⁵ The proinflammatory cytokines IL-1 α , IL-1 β and TNF- α may promote the sexual transmission of HIV-1 because they can enhance HIV-1 replication in infected cells by activating the HIV-1 proviral enhancer element located in the long terminal repeat (LTR) region of the HIV-1 proviral genome through activation of the NF- κ B nuclear factor.²⁸ Elevated levels of IL-1 β in cervicovaginal secretions have been associated with increased vaginal shedding of HIV-1.^{29,30} Cervicovaginal inflammation is also associated with increased levels of CC-chemokines which could recruit HIV-1 target cells to the vaginal epithelium or block HIV-1 infection through competition for CCR5 co-receptors.³¹

Proinflammatory cytokines and chemokines also provide a useful tool for evaluating cervicovaginal inflammation following the use of vaginal products. N-9, a commonly used vaginal spermicide, is now known to cause vaginal irritation and inflammation. However, clinical signs of overt cervicovaginal inflammation associated with N-9 use are difficult to observe and quantify.³² On the other hand, assessment of inflammatory cells and soluble markers of inflammation in cervicovaginal secretions from women following repeated vaginal exposure to N-9 demonstrated dramatic increases in granulocyte and macrophage cell concentrations, as well as in levels

Fig. 2 Effects of diaphragm with gel on concentrations of proinflammatory and immunologic markers in cervicovaginal secretions. Box plots representing levels of inflammatory factors (a) and chemokines and SLPI (b) in CVLs of women using diaphragms overnight for 14 days in combination with K-Y (□) ($n = 24$), AF (▨) ($n = 19$) or BG (■) ($n = 25$) at baseline (prior to diaphragm/gel use) and on day-8 (2–4 hrs after diaphragm/gel removal) and day-16 (24 hrs after diaphragm/gel removal). For each box, the horizontal lines, from bottom to top, represent the 25th, 50th (median) and 75th percentiles; the whiskers delineate the 10th and 90th percentiles; and the filled circles identify outlying values. Significant pair-wise comparisons represent results of *post hoc* analysis (Scheffé's *F*-tests) following significant ANOVA (*F*-test). Concentrations of MIP-1α, TNF-α and RANTES (data not shown) were consistently low and did not differ between time points or treatment groups. In Fig. 2a, significant reductions in IL-1α ($P < 0.01$), IL-1β ($P < 0.01$) and granulocyte elastase ($P < 0.05$) concentrations were observed in Day-8 CVLs compared with Baseline. Levels of IL-1α were significantly lower in Day-8 CVLs when compared with Day-16 ($P < 0.05$), and levels of granulocyte elastase were significantly lower in Day-16 CVLs when compared with Baseline ($P < 0.05$). All data were pooled across treatment groups. In Fig. 2b, levels of IL-8 were lowest in Day-8 CVLs from the BG treatment group, and concentrations of SLPI were significantly lower in both Day-8 ($P < 0.0001$) and Day-16 ($P < 0.01$) CVLs from the BG treatment group when compared with Baseline. Although there was a statistically significant interaction for MIP-1β, 84% of the MIP-1β values were below the detection limit, and the measured values were low, indicating that this effect is probably biologically insignificant (data not shown).

of granulocyte elastase, proinflammatory cytokines (IL-1α and β) and chemokines (IL-8, RANTES, MIP-1β).¹⁰ In animal vaginal toxicity models, levels of proinflammatory cytokines in vaginal lavages correlated with clinical signs of vaginal irritation/inflammation and provided a sensitive and informative measure of inflammation.^{17,18}

This study was designed to be conducted over a 14-day interval starting on day-5 of the menstrual cycle to avoid menses, which is associated with elevated levels of inflammatory markers in cervicovaginal secretions.³³ The baseline values amongst our study subjects are consistent with reported normal values from other studies.^{10,23} Previous studies have

shown that concentrations of many of the cervicovaginal inflammatory and immune markers remain relatively constant between day-5 and day-25 of the menstrual cycle.^{33–35} Because immunoglobulin (IgG, IgA and IgM) levels fluctuate in cervical mucus and CVLs between day-7 and day-14 of the menstrual cycle,³⁶ immunoglobulin levels were not monitored in this study.

We investigated the possibility that the microbicide and placebo gels interfere with the detection systems used in this study. Low pH (≤ 4) and high concentrations ($\geq 10\%$) of AF and BG inhibited the Bio-Plex/ELISA assay systems. Unfortunately, it was not possible to measure concentrations of AF and BG in individual CVLs to adjust for the inhibitory effects, but we took several steps to reduce the effects of product interference: (i) CVLs were obtained 2–4 hrs (day-8 sample) and 24 hrs (day-16 sample) after the removal of diaphragm with gel to allow for product clearance from the vagina, (ii) the pH of the CVLs was adjusted by the addition of PBS (pH 7.0), and (iii) CVLs were tested at 1:1, 1:10 and 1:100 dilutions in assays where concentrations were expected to be more than 100-fold times the lower limit of detection (i.e., IL-8, SLPI and granulocyte elastase) to further adjust for inhibitory effects of product. Of all the assays, the chemokine assays (IL-8, RANTES and MIP-1 α) were most inhibited by the microbicide gels.

Concentrations of inflammatory markers were not elevated in CVLs after daily use of diaphragm with K-Y or microbicide gel for 8 or 14 days. These data, in combination with the clinical observations from this study indicating lack of clinical signs of cervicovaginal inflammation in these subjects after diaphragm/gel use,¹⁶ provide evidence that daily use of diaphragms with K-Y or microbicide gel for up to 2 weeks does not induce cervicovaginal inflammation. IL-1 α and β concentrations were reduced in day-8 CVLs from the AF treatment group, and IL-8 and granulocyte elastase levels were reduced in day-8 samples from the BG treatment group. The day-8 samples were collected 2–4 hrs after diaphragm/gel use, and it is likely that they contained concentrations of residual gel that could have interfered with the detection assays.

Concentrations of SLPI were dramatically reduced in the BG treatment group at both day-8 and day-16 time points. Median SLPI concentrations for the BG group on day-8 and day-16 were 23% and 33% of the baseline median value for this group. Whereas the SLPI measurement in day-8 samples may have

been inhibited by residual gel, this is less likely for the day-16 sample, which was collected 24 hrs after removal of the diaphragm and gel. None of the other markers were reduced in day-16 samples from the BG-treated group, including IL-8, the assay most inhibited by BG, providing further evidence that the lower SLPI concentrations were caused by a biologic effect and not an assay artifact. The decrease in SLPI concentration suggests that daily use of diaphragms with BG for extended periods of time could have an adverse effect on cervicovaginal physiology. SLPI is constitutively secreted by vaginal and cervical epithelial cells and is thought to play an important role in innate immune defense of the lower female genital tract.²⁶ SLPI inhibits HIV infection *in vitro*,³⁷ and levels of SLPI in mucosal secretions have been inversely associated with the risk for perinatal HIV-1 transmission³⁸ and HIV-1 transmission through breast milk.³⁹ Furthermore, we observed reduced concentrations of SLPI in cervicovaginal secretions of women who used N-9,¹⁰ and N-9 use has been associated with increased HIV-1 transmission in several clinical studies.⁹ Therefore, the preliminary evidence provided by this study that diaphragm/BG use may adversely affect production of SLPI by the cervicovaginal mucosa is of concern and warrants further investigation. Our pilot sub-study, which also detected a significant decrease in lysozyme concentration following BufferGel™ use, highlights this concern.

To our knowledge, this study is the largest to date on cervicovaginal inflammatory markers in women using microbicide gels. The descriptive statistics derived from the baseline data, which describe median values and ranges of proinflammatory cytokine, chemokine, granulocyte elastase and SLPI concentrations in CVLs from 81 healthy untreated subjects during the proliferative phase of the menstrual cycle, provide a solid foundation of normative values for this and future studies on vaginal inflammation. The results of this study suggest that short-term daily use of the diaphragm with K-Y or non-irritating microbicide gel compounds does not induce cervicovaginal inflammation. However, further studies are needed to ensure that this holds true in research studies with larger sample sizes, in clinical practice with longer intervals of product use, and in diverse populations of women. It will also be important to continue to study the effects of microbicides, with or without diaphragms, on natural immune defense mechanisms in the genital tract.

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