The Effect of Commonly Used Excipients on the Epithelial Integrity of Human Cervicovaginal Tissue

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Abstract

Pharmaceutical excipients are widely used in vaginal drug products. The epithelial integrity of the cervicovaginal tissue is important for HIV-1 prevention. However, the effects of excipients on cervicovaginal epithelium remain unknown. This study aims at assessing the effects of vaginal product excipients on the integrity of human cervicovaginal epithelium and on a lead HIV prevention antiretroviral drug, tenofovir (TFV). In the current study, nine excipients commonly used in vaginal formulations were incubated for 6 h with excised human ectocervical tissue. The effects of the excipients were examined by measuring the transepithelial electrical resistance (TEER), epithelial morphology, paracellular/transcellular permeability, and cell viability. The efficacy of TFV for preventing HIV-1 infection in the *ex vivo* cultured ectocervix was also tested. We found that disodium ethyl-enediaminetetraacetate (EDTA), sorbic acid, and benzoic acid had no effect on the tissue TEER. Butylated hydroxyanisole, glycerin, propylene glycol, methylparaben, and propylparaben slightly to moderately decreased tissue TEER, whereas citric acid significantly decreased the TEER in a time-dependent manner. Tissue morphology observed post-exposure strongly correlated with TEER data; however, a less strong correlation was observed between paracellular permeability and TEER data after exposure to different excipients. In addition, treatment with EDTA, methylparaben, and propylene glycol at tested levels had no effect on the efficacy of TFV in preventing tissue HIV-1 infection. In conclusion, the combined measurements of TEER, morphology, permeability, and viability using human cervicovaginal tissue represent a clinically relevant platform for safety evaluation of excipients and formulated products for HIV-1 prevention.

Keywords: excipients, cervicovaginal epithelium, integrity, TEER, permeability, tenofovir

Introduction

EXCIPIENTS ARE CHEMICALS that help formulate the active pharmaceutical ingredient (API) into ready-for-use drug products. Depending on their role in pharmaceutical products, excipients can be categorized as chelators, antioxidants, solvents/cosolvents, preservatives, etc. They can help increase the solubility/compatibility/patient acceptability of the API and help maintain the physicochemical stability of the API in the formulated products.¹⁻⁴ A number of excipients with diverse functions are frequently used in vaginally administered drug products, including the vaginal microbicides tested in preclinical and clinical studies for topical preexposure prophylaxis (PrEP) of sexually transmitted HIV-1.⁵⁻⁸

In the design and development of vaginal formulations, the effect of formulation ingredients on the integrity of the cervicovaginal epithelium should be carefully evaluated. This is

especially true in the formulation of vaginal microbicides, which are intended for the prevention of sexually transmitted infections (STIs). The ectocervical and vaginal epithelium is composed of multi-layer stratified squamous epithelial cells with tightly sealed intercellular junctions at the basal epithelial layer.⁹ The intact epithelium functions as a natural barrier against STIs such as HIV-1.^{10,11} If a formulation excipient denudes epithelial layers or opens intercellular junctions, it will compromise the epithelial integrity and potentially increase the risk of viral penetration and infection.¹²

The importance of epithelial integrity in HIV prevention was exemplified by the failures of two phase III trials testing the nonoxynol-9 (N-9) vaginal gel (COL-1492) and the 6% cellulose sulfate (CS) gel. In these trials, N-9 and CS were tested as microbicide candidates for HIV-1 prevention, but they were found to increase HIV infection rate.¹³⁻¹⁵ A key reason for this unsatisfactory outcome was the disruptive

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effect of both agents on cervicovaginal epithelial layers.^{5,12,14–16} Therefore, when formulating vaginally administered drug products for the prevention of STIs, the epithelial safety of the formulation ingredients must be thoroughly evaluated.

Despite extensive utilization of excipients in vaginal drug products for PrEP, there is limited understanding regarding the safety of vaginal product excipients in the context of HIV-1 prevention. In clinical trials, “universal HEC gel” has been used in the placebo arm, which contains several pharmaceutical excipients. The safety of many vehicles and single excipients were either unknown or only grossly evaluated. Currently, robust and reproducible efficacy has not been observed in the microbicide clinical trials for the prevention of sexually transmitted HIV-1. Safety concerns have been raised regarding the formulation components, in addition to APIs.¹⁷

There is some evidence suggesting that excipients may interact with the human cervicovaginal epithelium. Some hyperosmolar over-the-counter lubricant gels have been found to reduce the tissue viability and to result in epithelial fracture/sloughing in an ectocervical explant model.¹⁸ Several other tissue explant and mouse studies reported that treatment with excipients that are frequently used in vaginal products could alter the cervicovaginal tissue integrity and even increase the rate of vaginal herpes simplex virus 2 (HSV-2) transmission.^{19–21} McClelland *et al.* evaluated the effect of vaginal washing on the incidence of HIV-1 infection in 1,270 African women between 1993 and 2003. They found that women who partake in vaginal washing, by vaginal douching or washing with water and soap, had higher HIV acquisition risk as compared with women who did not perform vaginal washing. In addition, women who performed vaginal washing with soap had a higher risk for HIV-1 infection compared with those who used water alone.²² Clearly, the safety profile of product components in addition to the API needs to be better understood to inform the product development, clinical trial design, and data interpretation. Toward this goal, it is necessary to examine the effect of various extensively used excipients on cervicovaginal tissue in a clinically relevant model and to determine whether excipients influence active product efficacy.

Therefore, this study aims at examining the effect of commonly used excipients on the epithelial integrity of surgically excised human ectocervical epithelium. This tissue is

anatomically similar to the vaginal epithelium, and it represents the cervicovaginal epithelium in a product use setting. In our study, the ectocervical epithelium that was excised for benign conditions was exposed to nine excipients: disodium ethyl-enediaminetetraacetate (disodium EDTA), citric acid, butylated hydroxyanisole (BHA), sorbic acid, glycerin, propylene glycol, methylparaben, propylparaben, and benzoic acid. These excipients are commonly used in vaginal formulations, and some of them are reported to affect certain aspects of the epithelial barrier.^{19,20} The effect on epithelial integrity was evaluated by analyzing the transepithelial electrical resistance (TEER), tissue morphology (hematoxylin & eosin [H&E] staining), paracellular permeability (mannitol flux), and cell viability ([1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] (MTT) assay), with or without excipients. Finally, the effect of excipients on tissue susceptibility to HIV-1 infection was tested in an explant model by using an *ex vivo* cultured human ectocervix.

To our knowledge, this is the first article investigating the effect of excipients on the integrity of intact human cervicovaginal tissue directly excised from patients. The results obtained in this study could inform the design and development of various kinds of vaginally administered products.

Materials and Methods

Materials

The excipients tested in this study are summarized in Table 1. D-[1-14C]-Mannitol (¹⁴C-mannitol) and L-[4-3H]-Propranolol (3H-propranolol) were obtained from Perkin-Elmer Life Sciences, Inc. (Boston, MA). Disodium EDTA, benzoic acid, citric acid, BHA, propylene glycol, sorbic acid, glycerin, methylparaben, propylparaben, and N-9 were obtained from Spectrum Chemical Manufacturing Corp. (New Brunswick, NJ). Tenofovir (TFV) drug substance was obtained from the International Partnership for Microbicides. TFV gel is composed of 1% TFV that is incorporated into a formulation containing a gelling agent (hydroxyethylcellulose [HEC]), glycerin, EDTA, citric acid, and the preservatives methyl and propyl parabens. Universal placebo HEC gel was previously described.²³ All other chemicals and solvents were obtained from Fisher Scientific, Inc. (Fair Lawn, NJ). All the excipients and permeability markers were dissolved in

TABLE 1. EXCIPIENTS USED IN THE STUDY^a

Class	Excipient	Commonly used concentration range (%) ^b	Formulation type
Chelator	Disodium EDTA	0.01–0.1	Emulsion, cream, gel
Antioxidant	Citric acid	0.1–2.0	Emulsion, cream, sponge
	BHA	0.005–0.02	Emulsion, cream, ointment, suppository
	Sorbic acid	0.05–0.2	Gel, sponge
Solvent/cosolvent	Glycerin/glycerol	5–15	Emulsion, cream, gel, suppository
	Propylene glycol	5–80	Emulsion, cream, gel, suppository, tampon
Preservative	Methylparaben	0.1–0.18	Emulsion, cream, gel, suppository
	Propylparaben	0.02–0.1	Emulsion, cream, gel, suppository
	Benzoic acid	0.1–0.2	Emulsion, cream, sponge

^aThe information was derived from Inactive Ingredients in the FDA-Approved Drugs Database (www.accessdata.fda.gov/scripts/cder/IIG/).

^bCommonly used concentration range in vaginal formulations.

BHA, butylated hydroxyanisole; EDTA, ethyl-enediaminetetraacetate; FDA, Food and Drug Administration.

TABLE 2. SUMMARY OF TEST RESULTS

Excipient (tested concentration, w/v)	TEER (% of initial value) ^a	Tissue morphology ^b	Fold change in P_{app} -man ^c	Viability (%) ^d
Disodium EDTA (0.05%)	107.2 ± 4.3	+	1.2	NA
Disodium EDTA (0.1%)	96.7 ± 7.0	+	1.5	96.0 ± 26.7
Disodium EDTA (1%)	92.5 ± 15.0	+	5.0*	82.5 ± 49.4
Citric acid (1.14%)	57.5 ± 7.3**	–	7.6**	10.5 ± 13.3**
BHA (0.02%)	90.4 ± 0.1*	+/-	1.2	80.6 ± 45.5
Sorbic acid (0.125%)	100.1 ± 0.6	+	0.7	44.8 ± 21.7*
Glycerin/glycerol (10%)	85.2 ± 12.3	+/-	2.5	86.5 ± 21.0*
Propylene glycol (10%)	81.3 ± 6.4*	+/-	6.6*	84.5 ± 32.0
Methylparaben (0.14%)	86.2 ± 4.6*	+/-	3.9**	102.8 ± 16.2
Propylparaben (0.06%)	81.2 ± 1.3**	+/-	1.2	79.2 ± 27.5
Benzoic acid (0.15%)	98.5 ± 1.5	+	0.7	52.6 ± 39.1
N-9 (4%)	41.0 ± 6.1**	–	11.0**	59.0 ± 5.4**

^aThe TEER value was calculated by normalizing the TEER at the 6-h time point to the initial TEER of the same tissue sample at time zero. Data represent the mean ± SD of at least three independent experiments.

^bThe tissue morphology was arbitrarily classified as intact or disrupted. +, all the examined tissue samples showed intact epithelial layers; –, all the examined tissue samples showed disrupted epithelial layers; +/-, heterogeneous results among the examined tissue samples were observed.

^cThe fold change in P_{app} -man was calculated by normalizing the P_{app} -man with excipients to the P_{app} -man with PBS. TEER and viability data represents the mean ± SD of at least three independent experiments.

^dThe viability % was calculated by normalizing the MTT value obtained with excipients to the MTT value obtained with PBS (100%). Data represent the mean ± SD of at least three independent experiments.

* $p < .05$, ** $p < .01$.

N-9, nonoxynol-9; NA, not available; PBS, phosphate-buffered saline; SD, standard deviation; TEER, transepithelial electrical resistance.

phosphate-buffered saline (PBS), pH 7.4, at the concentrations denoted in Table 2.

Ethics statements

All human ectocervical tissues used in this study were obtained through the University of Pittsburgh Health Sciences Tissue Bank as per approved IRB protocol PRO09110431. All tissues were de-identified and collected through an Honest Broker.

Human ectocervical tissue

Human ectocervical tissue was obtained from a total of 87 patients (30–55 years old) undergoing hysterectomy for indications that were unrelated to the present study. Tissues were held at 4°C in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc., Herndon, VA) during transfer from the surgical room to the lab, which occurred within 5 h of surgical excision. The tissues were used immediately on procurement or snap-frozen and stored at –80°C until needed. Methanol was used to freeze tissue as previously described.²⁴ Briefly, crushed dry ice was added to methanol (1:1 w/v) to form a slurry. The tissue was placed in a re-sealable specimen storage bag (Fisher Scientific, Inc.) and immersed in the slurry for 1–2 min until it was frozen. Tissue was then stored at –80°C. To thaw, the specimen storage bag containing tissue was placed in a 37°C water bath for 5–10 min until it was thawed.

All ectocervical tissues used in this study were classified as histologically normal based on H&E staining results. In addition, our preliminary studies demonstrated that freeze-thawed and fresh ectocervical tissues did not differ in basal levels of TEER, epithelial morphology, and mannitol permeability (data not shown). The epithelial layer was isolated by using a Thomas–Stadie–Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ), and epithelial thickness was measured before the studies as described elsewhere.²⁴

Electrophysiological measurements

TEER was measured by using an Ussing Chamber system (Physiologic Instruments, Inc., San Diego, CA). The excised ectocervical epithelia were mounted in the Ussing chamber and equilibrated for 60 min with PBS that was filled into both hemichambers. TEER monitoring was initiated by replacing the PBS in the epithelial side hemichamber with excipient solution (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/aid). For TFV or HEC gel treatment, gels were diluted with PBS and then placed into the epithelial side hemichamber. All tissue used in this study was fresh human ectocervical tissue, and the exposed tissue area in the chamber system was 0.26 cm². Hemichambers were bubbled continuously with environmental air to provide oxygenation and stirring of the solutions. The mounted tissue samples were exposed periodically to a 1 mV bipolar pulse of 100-ms duration. The resultant short circuit current (I_{sc}) was recorded in a real-time manner throughout the 6-h incubation period, with a multichannel voltage clamp through a pair of Ag/AgCl electrodes that were immersed in 3% (w/v) agarose gel containing 3.0 M of potassium chloride. Data were acquired and processed at 0.1–1 Hz (Acquire and Analyze Rev II software, version 3.1; Physiologic Instruments, Inc.). TEER ($\Omega \cdot \text{cm}^2$) was determined by using the current deflections with Ohm's law. The TEER at the 6-h time point was compared with the initial value (shown as Time 0) to indicate the effect of excipient incubation on TEER.

Histological evaluations

Histological evaluation was conducted on all tissue specimens from the TEER experiments as previously described.^{5,25} Briefly, tissue pieces were obtained both before and after the excipient solution exposure, fixed in acid alcohol or formalin, and embedded in paraffin. H&E staining

was performed on sections of 5 μm thickness. Pictures were taken by using a Zeiss (Genna, Germany) Axioskop 40 microscope with AxioVision Software.

Permeability studies

The permeability studies were performed by using a Franz Cell system as previously described,²⁴ with minor modifications. The 7 mm Franz cells (Permeagear, Inc., Hellerstown, PA) were water-jacketed, and the temperature was maintained at $37^\circ\text{C} \pm 0.5^\circ\text{C}$ throughout the experiment. The receptor chamber was filled with 5.0 ml PBS (pH 7.4) solution and continuously stirred. The excised epithelia (fresh or freeze-thawed) were placed in the Franz Cell with the epithelial side facing the donor solution, which yielded a diffusion area of 0.385 cm^2 . The tissue was equilibrated with PBS (pH 7.4) in the donor compartment for 15 min before the study. The study was initiated by replacing the equilibrating PBS with 450 μl of ^{14}C -mannitol ($12.5\text{ }\mu\text{Ci}\cdot\text{ml}^{-1}$) or ^3H -propranolol ($1.0\text{ }\mu\text{Ci}\cdot\text{ml}^{-1}$), with or without excipients. After the initial experimental setup, a 50 μl sample was taken from the donor compartment to measure the baseline exposure level. Aliquots of 200 μl were removed from the receptor compartment at 15, 30, 45, 60, 120, 180, 240, 300, and 360 min after initiation, and they were replaced with fresh PBS (pH 7.4) to maintain sink conditions. Radioactivity was measured by using a Wallac 1409 DSA Liquid Scintillation Counter (Perkin-Elmer Life Sciences, Inc., Boston, MA). The cumulative radioactivity in the receptor compartment, as a percentage of the initial radioactivity in the donor compartment, was plotted over time to reflect the permeation profile of ^{14}C -mannitol through the epithelial layers. Apparent permeability coefficient, P_{app} , was calculated as follows:

$$P_{\text{app}} = \frac{dQ}{dt \cdot A \cdot C_d} \times 60 \text{ (cm} \cdot \text{s}^{-1}\text{)},$$

where dQ/dt was the slope of the curve of Q (amount in cpm) versus t (time in min), A was the surface area (cm^2) of the

tissue exposed to the solution, and C_d was the initial radioactivity of the ^{14}C -mannitol in the donor compartment ($\text{cpm}\cdot\text{ml}^{-1}$).

Tissue viability analysis

The MTT assay for epithelial viability was performed by using a previously reported procedure with modification.²⁶ In brief, the freshly excised tissues used in the permeability experiments were collected after the final sampling time point, and they were incubated with 0.2 ml of 0.5 mg/ml MTT that was dissolved in PBS for 3–4 h at 37°C . The MTT solution was then discarded, and the tissues were weighed. The formazan formed in the cells was extracted overnight in 2 ml of n-propanol, and the absorbance was measured at 540 nm with readings at 620 nm serving as blank, in a regular UV-spectrophotometer. In this study, tissue viability was calculated as the $\text{Abs}\cdot\text{mg}^{-1}$ compared with the control tissues that were incubated with PBS in the permeability experiment. Previously freeze-thawed tissue was not used in the MTT assay.

Ex vivo efficacy study

The normal human ectocervical tissues were used in this study. Polarized explant cultures were set up as previously described.^{5,26} Briefly, the explant was placed with the luminal side up in a transwell system. The edges around the explant were sealed with MatrigelTM (BD Biosciences, San Jose, CA). The explants were maintained with the luminal surface at the air–liquid interface. The lamina propria was immersed in medium for ectocervical explants. Cultures were maintained at 37°C in a 5% CO_2 atmosphere. Then, $2\times\text{TFV}$ or TFV/excipient solution was prepared in complete culture medium, and 100 μl of $2\times\text{TFV}$ or TFV/excipient solution was added to the apical side of the explants, which was followed by adding 100 μl of 5×10^4 tissue culture 50% infective dose (TCID_{50}) of HIV-1_{BaL}. The final concentration of TFV was 500 μM , and the excipient concentrations were listed in Table 3. Twenty-four hours after application, the

TABLE 3. CALCULATION OF PERMEABILITY PARAMETERS OF ^{14}C -MANNITOL AND ^3H -PROPRANOLOL^a

Excipient/control	Flux-man ($\text{cpm}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$)	$P_{\text{app-man}} \times 10^6$ ($\text{cm}\cdot\text{s}^{-1}$)	Flux-prop ($\text{cpm}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$)	$P_{\text{app-prop}} \times 10^6$ ($\text{cm}\cdot\text{s}^{-1}$)
PBS	22.5 $\pm$ 23.3	0.5 $\pm$ 0.2	70.8 $\pm$ 25.0	3.9 $\pm$ 1.8
Disodium EDTA (0.05%)	14.1 $\pm$ 3.9	0.6 $\pm$ 0.1		
Disodium EDTA (0.1%)	17.9 $\pm$ 4.3	0.8 $\pm$ 0.2		
Disodium EDTA (1.0%)	41.6 $\pm$ 41.4	2.6 $\pm$ 1.5*	91.8 $\pm$ 37.3	4.8 $\pm$ 2.7
Citric acid (1.14%)	104.9 $\pm$ 41.5**	3.9 $\pm$ 2.3**		
BHA (0.02%)	113.9 $\pm$ 104.9	0.6 $\pm$ 0.4		
Sorbic acid (0.125%)	17.1 $\pm$ 17.3	0.4 $\pm$ 0.4		
Glycerin/glycerol (10%)	30.7 $\pm$ 28.4	1.3 $\pm$ 1.1	113.9 $\pm$ 54.3	5.1 $\pm$ 3.2
Propylene glycol (10%)	76.5 $\pm$ 44.0	3.4 $\pm$ 2.0*	94.6 $\pm$ 65.8	4.0 $\pm$ 3.0
Methylparaben (0.14%)	83.4 $\pm$ 45.2*	2.0 $\pm$ 0.3**	45.0 $\pm$ 9.0	2.4 $\pm$ 0.3
Propylparaben (0.06%)	47.4 $\pm$ 44.7	0.6 $\pm$ 0.6		
Benzoic acid (0.15%)	54.9 $\pm$ 7.4*	0.4 $\pm$ 0.1		
N-9 (4.0%)	111.2 $\pm$ 14.0**	5.6 $\pm$ 0.8**		

Data represent the mean $\pm$ SD of at least three independent experiments.

^aThe effect on ^{14}C -mannitol permeability was studied for every excipient, whereas the effect on ^3H -propranolol permeability was studied only for those excipients that caused opposite changes in TEER and ^{14}C -mannitol permeability.

* $p < .05$, ** $p < .01$.

Flux-man, flux of ^{14}C -mannitol; $P_{\text{app-man}}$, apparent permeability coefficient of ^{14}C -mannitol; Flux-prop, flux of ^3H -propranolol; $P_{\text{app-prop}}$, apparent permeability coefficient of ^3H -propranolol.

explants were washed and fresh culture medium was applied to the basolateral compartment. Every 3–4 days over a 3-week period, supernatant was collected and stored at -80°C for further analysis. Fresh medium was replaced in the basolateral compartment. At day 21, the supernatant was tested for HIV-1 replication by using the p24gag enzyme-linked immunosorbent assay (ELISA; Perkin-Elmer Life and Analytical Sciences, Inc., Waltham, MA). All tissue samples used in this study were from premenopausal women; however, the information regarding donors' menstrual cycle stage or hormone use was not available.

Statistical analysis

Quantitative data were expressed as mean $\pm$ standard deviation (SD). Statistical analyses were conducted by using Student's *t* test, and $p < .05$ was considered statistically significant. The differences between multiple groups, at various time points, was performed by using the one-way analysis of variance (ANOVA). All error bars represent SDs.

Results

Selection of excipients and determination of experimental conditions

The excipients examined in this study were as follows: disodium EDTA, citric acid (CA), sorbic acid (SA), benzoic acid (BA), butylated hydroxyanisole (BHA), glycerin, propylene glycol (PG), methylparaben (MP), and propylparaben (PP). The categorization, commonly used concentration, and chemical structure of these excipients are listed in Table 1. These excipients have been widely used in vaginally administered products. For example, glycerin, propylene glycol, sorbic acid, methylparaben, and propylparaben were used in the 1% TFV gel⁵ and 0.05% dapivirine gel²⁷ that were evaluated in clinical trials such as CAPRISA 004, VOICE, FACTS 001, IPM003, and IPM005B trials.^{8,28,29} Although identified as generally-regarded-as-safe excipients by the Food and Drug Administration (FDA), several of these excipients were reported to affect the epithelial barrier in human cell culture or animal models.^{18–21} Excipients chosen for evaluation represent a variety of functional categories and diverse chemical structures. The excipient concentration used in this study (Table 2) was determined based on the following considerations: Disodium EDTA is widely reported to decrease the integrity of intestinal cell monolayer (Caco-2)³⁰ and excised intestinal epithelium,³¹ so three concentrations (0.05%, 0.1%, and 1%) were chosen to cover the relevant levels of use. For all other excipients, a single concentration was studied. The concentration chosen was based on the common level of use in vaginal products. Due to the dilution by cervicovaginal fluid and other body fluids, the concentration of excipients in cervicovaginal fluid is usually lower than that in administered drug products, and the results obtained in this study could indicate the effect of those excipients at the highest possible *in vivo* concentrations. The incubation time for all experiments was 6 h.

The effect of excipients on cervicovaginal epithelium TEER

The effect of excipients on TEER was monitored in a real-time manner by using an Ussing Chamber instrument. As

shown in Table 2 and Figure 1, the negative control PBS-treated tissues showed stable TEER throughout the 6-h assay period; whereas the positive control N-9 (4%) resulted in an approximate 40% decrease in TEER after 3 h incubation, and a 59% decrease after 6 h incubation. The discrepancy in TEER between PBS and N-9 groups appeared as early as 20 min post exposure.⁵ These results were consistent with previous clinical and preclinical observations with PBS and N-9, confirming the ability to distinguish between benign and disruptive treatments by using this experimental setup. For tested excipients, disodium EDTA (0.05%, 0.1%, 1%), sorbic acid (0.125%), and benzoic acid (0.15%) did not show any effect of ectocervical epithelium on TEER throughout the 6-h assay period when compared with PBS. Methylparaben (0.14%), propylparaben (0.06%), glycerin (10%), propylene glycol (10%), and BHA (0.02%) exposure resulted in a slightly decreased TEER value. This decrease was negligible at 3 h, and it was between 10% and 20% at the 6 h time point. On the other hand, exposure to citric acid (1.14%) resulted in a significant decrease in TEER (25% at 3 h and 43% at 6 h).

The effect of excipients on epithelial morphology

The effect of the excipients on epithelial morphology was examined by using H&E staining, which includes stripping of the epithelium from the lamina propria, loss of nuclei, and disruption of the lamina propria. It was anticipated that an intact multi-layer epithelium would be seen for those tissues with unchanged TEER; whereas detachment of uppermost epithelial layers or total denudation of the epithelium from lamina propria would be seen for tissues with a significantly decreased TEER. In this study, for each staining slide, three to five pictures were taken at different tissue sites and representative pictures were chosen. As shown in Table 2 and Figure 2, the morphology of PBS- and N-9-treated tissues correlated well with their TEER values obtained. PBS-treated tissues maintained an intact stratified squamous epithelium, which comprised up to 40 epithelial layers; no swelling or necrosis of the epithelial cells could be observed. In contrast, the N-9-treated tissues lost multiple superficial layers, which was a result of the extensive disruption of epithelial cells and the intercellular tight junctional proteins.

Among the tested excipients, citric acid exposure resulted in obvious damage to the ectocervical epithelium. This epithelial damage coincided with an observed decrease in TEER. The effects of BHA (0.02%), methylparaben (0.14%), propylparaben (0.06%), glycerin (10%), and propylene glycol (10%) on tissue morphology were heterogeneous. Exposure to these excipients resulted in inconsistent effects on the epithelium, with some tissues unchanged from the untreated control and with others showing epithelial fracture. This intra-group variability was probably due to inter-individual variability in tissue sensitivity to toxic stimuli. Tissues treated with disodium EDTA (0.1%), sorbic acid (0.125%), and benzoic acid (0.15%) showed an unchanged morphology after 6 h of incubation, which was comparable to the PBS group.

The effect of excipients on paracellular permeability

The effect of excipients on paracellular permeability was measured by using ³H-mannitol. The purpose of this

FIG. 1. Effect of excipients on TEER in the human ectocervical epithelium. Epithelium was excised from ectocervical tissue and equilibrated with PBS solution for 1 h before the TEER measurement. The TEER was monitored at real time during exposure to PBS (control) or different excipient PBS solution (in Table 2) for 6 h. The TEER value was calculated as described under the Materials and Methods section and normalized to the percentage of the initial TEER value (TEER value after equilibration). The results shown are the means $\pm$ SD for at least three independent determinations (three tissues from patients). PBS, phosphate-buffered saline; SD, standard deviation; TEER, transepithelial electrical resistance.

experiment was to confirm TEER observations obtained. For the excipients that decreased tissue TEER and damaged epithelial layers, an increase in the apparent permeability coefficient ($P_{app-man}$) would be expected; for those excipients that did not affect TEER and tissue morphology, e.g., disodium EDTA (0.05%, 0.1%, 1%), the $P_{app-man}$ would be expected to remain unchanged. As shown in Table 2 and Figure 3, compared with the PBS-treated tissues, N-9 (4%) caused an 11-fold increase in $P_{app-man}$. The slope of the flux curve increased with time, reflecting the gradual disruption of cell layers by the N-9 and a corresponding time-dependent increase in epithelial permeability.

Among all the tested excipients, citric acid (1.14%) resulted in the largest increase in $P_{app-man}$ (7.6-fold compared with PBS treatment with $p < .01$). This finding is consistent with a dramatic decrease and a significant disruption of the cervicovaginal epithelium in TEER, after a 6-h exposure (Fig. 2). The 1% and 0.1% disodium EDTA significantly increased $P_{app-man}$ by 5.0- and 1.5-fold compared with PBS treatment, which appeared paradoxical to the observation that EDTA had no effect on TEER and epithelial morphology (Table 2). The effect of disodium EDTA on $P_{app-man}$ appeared to be concentration dependent (Fig. 3). Propylene glycol, glycerin, and methylparaben caused a moderate-to-large increase in the $P_{app-man}$, which was consistent with but not proportionate to the change in TEER and morphology that they induced. BHA and propylparaben slightly increased $P_{app-man}$, which was consistent with moderately decreased epithelial TEER and slightly altered epithelial morphology.

The effect of select excipients on transcellular permeability

Since disodium EDTA, glycerin, propylene glycol, and methylparaben increased the paracellular permeability, the effect of these four excipients on transcellular permeability was also examined by using the transcellular probe 3H -propranolol. None of the four excipients significantly altered the propranolol permeability coefficient ($P_{app-prop}$) at the concentrations tested (Table 3).

The effect of excipients on epithelial cell viability

The MTT-based colorimetric assay was used to measure the effect of excipients on epithelial viability. In live cells, the MTT reagent can be metabolized by mitochondrial reductase into colored formazan products. Therefore, a significant decrease in MTT value indicates death of cells and/or inhibition of cellular metabolic function and an increased MTT value often suggests activated mitochondrial metabolism. The purpose of this assay in the present study was to examine the effect of excipients on epithelial cell viability and to correlate these findings with those of TEER, morphology, and permeability studies. If the excipient disrupted epithelial cells, the MTT value of treated tissue would be expected to be low when compared with the control PBS-treated tissues. As shown in Table 2, the MTT value of the N-9-treated tissues was $59\% \pm 5.4\%$ of the PBS-treated tissues. Among the excipients, exposure to citric acid (1.14%) resulted in a dramatic 90% decrease in MTT value. This decrease is consistent with the marked TEER and histological changes

FIG. 2. The effect of excipients on tissue morphology. Human cervicovaginal epithelia used in the TEER and/or permeability studies were subjected to the H&E staining. All tissue samples were incubated with excipient solution, PBS, or N-9, for 6 h at 37°C with oxygenation. For each excipient, at least three treated tissue samples were used for the staining and analysis. The tissue morphology was arbitrarily classified as intact (+) or disrupted (–) to indicate the effect of excipients in Table 2. For excipients that displayed homogeneous effect on tissues (disodium EDTA at all concentrations, citric acid, BHA, benzoic acid), only one representative picture was shown. For the excipients that displayed heterogeneous effects (sorbic acid, glycerin, propylene glycol, methylparaben, and propylparaben), both the intact morphology and disrupted morphology were shown. Magnification, 20×. BHA, butylated hydroxyanisole; EDTA, ethyl-enediaminetetraacetate; H&E, hematoxylin and eosin; N-9, nonoxynol-9.

FIG. 3. The effect of excipients on ^{14}C -mannitol transport across human ectocervical epithelium. The time course of ^{14}C -mannitol permeation was shown as the amount appearing in the acceptor chamber that was normalized to the initial amount applied to the donor chamber. The calculation of permeability parameters was described in Table 3. Each data point represented the mean $\pm$ SD of at least three independent tissues.

observed with citric acid exposure. Sorbic acid also induced a significant decrease in the MTT value. Conversely, tissue exposure to disodium EDTA at 0.05%, 0.1%, and 1%, BHA (0.02%), methylparaben (0.14%), propylparaben (0.06%), glycerin (10%), or propylene glycol (10%) did not result in decreases in MTT.

The effect of topical gels on cervicovaginal epithelium TEER

The effect of vaginal TFV (1%) or universal placebo HEC gel on human ectocervical tissue TEER was monitored in a real-time manner by using an Ussing Chamber instrument. The TFV gel formulation was the same as that was used for several microbicide clinical studies, such as CAPRISA004 and VOICE.^{32,33} As shown in Figure 4, the negative control PBS-treated tissues showed stable TEER throughout the 6-h assay period, whereas the positive control N-9 (4%) resulted in a dramatic decrease in TEER after 6 h incubation. None of the tested TFV gel (1–10 and 1–5 diluted in PBS) or HEC gel (1–10 diluted in PBS) solution showed any effect on TEER of ectocervical epithelium throughout the 6-h assay period.

Ex vivo efficacy study

The effect of excipients on tissue integrity was further evaluated in an HIV challenge model by using excised human ectocervical tissue that was cultured *ex vivo*. The untreated control explants (HIV-1 only) peaked to 10^4 pg/ml on day 11 and were maintained at this level for the rest of the test period (Fig. 5). The TFV-treated explants showed decreased p24 in the culture supernatant, which was indicative of this anti-

retroviral drug in suppressing HIV replication. The p24 curves of the explants treated with TFV and the excipients (EDTA, methylparaben, and propylene glycol) closely resembled the curve of TFV-treated-only explants, with a few exceptions. The explants treated with TFV and PG displayed higher p24 levels than the TFV-treated explants, whereas all excipient-treated explants were less than 10-fold different than the HIV-1-only explants. Overall, there is a clear separation in the p24 curve pattern between the positive control group and the groups treated with excipients.

FIG. 4. The effect of TFV (1%) and HEC gel on TEER. The TEER was monitored at various time points during the 6 h exposure to PBS (negative control), N-9 (positive control), and different TFV (1%) and HEC gels diluted in PBS. The TFV and universal placebo HEC gels were diluted with PBS (1–5 or 1–10 dilution for TFV gel, and 1–10 dilution for HEC gel). Each data point represents the mean $\pm$ SD of tissue samples from at least three patients. HEC, hydroxyethylcellulose; TFV, tenofovir.

FIG. 5. The effect of the excipients on the *ex vivo* efficacy of TFV using human ectocervical tissue explant cultures. Tissues were exposed to TFV alone or with different excipient solutions and HIV-1 overnight, followed by washing and 21 days of culture. At the end of the study, the supernatant was tested for HIV-1 replication by using the p24gag ELISA. Each point represents mean $\pm$ SD of tissue samples from at least three patients. ELISA, enzyme-linked immunosorbent assay.

Discussion

This study utilized intact human ectocervical tissue directly excised from patients, which overcame the limitation of previous studies. Previously, Grammen *et al.* and Moench *et al.* evaluated the effect of excipients on cervicovaginal tissue by using *in vitro* cell lines and a mouse model.^{19,20} However, the baseline thickness of the epithelial layers, the types of epithelial cells present in the tissue, as well as the type and abundance of tight junction proteins are quite different between intact human cervicovaginal tissue and these preclinical models.^{34,35} These differences affect the interpretation of previous findings, and they limit their applicability toward optimizing clinically applied vaginal PrEP products. In our opinion, although these models are valuable tools for early-stage toxicity screening, the surgically excised human cervicovaginal tissue represents a more clinically relevant model for safety evaluation of excipients and formulated products.

Using our model, we have demonstrated that excipients that are generally regarded as safe could alter various aspects of cervicovaginal tissue integrity, including TEER, epithelial morphology, permeability, and viability. In addition, these effects are dependent on excipient concentration and the duration of treatment. At the concentrations tested in this study, disodium EDTA below 0.1%, sorbic acid, and benzoic acid did not compromise tissue TEER, morphology, and permeability. EDTA above 1% did not decrease TEER but significantly increased tissue permeability. In addition, BHA, glycerin, propylene glycol, methylparaben, propylparaben, and citric acid displayed time-dependent negative effects on more than one aspect of tissue integrity. A further evaluation of the effect of EDTA, PG, and MP using the *ex vivo* cultured human ectocervical tissues demonstrated that although these excipients altered TEER, morphology, paracellular permeability, and viability of excised human tissue to different

degrees, they did not exert significant impact on tissue susceptibility to HIV infection when used in conjunction with an active API. This was evidenced by the clear separation in the time-dependent p24 curves between excipient-treated tissues and positive control-treated tissues (Fig. 5). Taken together, these observations suggest that when evaluating the safety of excipients used in vaginal formulations for the purpose of HIV prevention, the commonly used *in vitro* testing methods (TEER, histological staining, paracellular permeability, viability) have limitations and the value of a single assay should not be overstated. A comprehensive analysis of the results from all these assays is more likely to reflect a clinically relevant evaluation of the excipient's safety. The observations reported in this study also suggest that the tested excipients except citric acid are safe to use for vaginal product development at tested concentrations.

The results from this study have implications for future PrEP studies. Despite widespread use of the excipients tested in this study, it has been reported that some commonly used excipients could alter the permeability of mannitol, thus raising the concern that these excipients could open the intercellular gates to facilitate HIV entry. Gali *et al.* further demonstrated that EDTA and glycerin could increase vaginal tissue susceptibility to viral infection (HSV-2) in a mouse model. We have demonstrated, using excised and cultured human tissues, that these excipients are generally safe at clinically relevant concentrations, although they may change the TEER, permeability, and viability to various extents. The apparent discrepancies between our results and other published studies may be attributable to the difference in models (human tissue vs. cell line or animals) and test conditions (e.g., concentration of excipient).

Exposure is a critical factor in determination of the excipient's safety. In our study, citric acid is found to harm tissue integrity in every aspect (TEER, morphology, permeability, viability). However, citric acid has been formulated into various vaginal products and no significant toxicity has been observed. In fact the TFV gels tested in our study also contain citric acid, and its clinical use is regarded as safe. This discrepancy is presumably caused by limited safety testing of clinically used vaginal products. The safety evaluation is often conducted in the population with regular product use. Under such conditions, the excipients exert their functions in the formulation (e.g., citric acid as antioxidant); thus, the amount of biologically reactive excipients available to the tissue might be less than their total amounts contained in the product. In addition, the excipients are usually gradually released from the product, and a significant amount of excipients might be entrapped in the cervicovaginal mucus. Moreover, the vaginal retention of most administered dosage forms, such as gel, film, tablet, and suppository, is short due to vaginal discharge. Therefore, under regular clinical use, tissue exposure to the product-released excipients might be lower than the concentrations tested in our experimental setting. However, the lessons learned from microbicide clinical trials highlight the necessity of thoroughly evaluating the safety of product components under all conditions that may be encountered by PrEP product users.

However, there is a variety of product- and patient-related factors that could result in higher-than-expected exposure to excipients. The excipient concentration in the product is frequently changed for better product properties. In the

context of PrEP, the demographics show that those at the highest vaginal infection risk are sex workers and those who have multiple sex partners. These people use the product much more frequently than the rest of the population, and their tissue exposure to excipients may be much higher than the majority of people who use the product less frequently. Indeed, the COL-1492 trial revealed that the sex workers used the N-9 gel >3.5 times per day, and the vaginal toxicity and increased HIV-1 acquisition that were associated with this particular subgroup were not observed among those who used the gel only once per day.¹³ In addition to dosing frequency, other patient-related factors such as reduced viscosity of cervicovaginal fluid could also result in elevated exposure of excipients. In this study, the 1% TFV vaginal gel was also found to have no effect on TEER and tissue morphology in our tissue model (data not shown), but a number of excipients contained in this product affected multiple aspects of tissue integrity when they were administered to the tissue as solutions. These effects, in our opinion, reflect the potential toxicity risk under the conditions of high-level product exposure. In these “enhanced exposure” scenarios, it is possible that even the excipients that are shown to be safe in our study could become harmful when their concentrations are markedly increased. Hence, our results can help PrEP researchers with future development and labeling of product formulations, and they can provide clues for interpreting the suboptimal clinical trial efficacy results.

This study revealed that excipients could result in inconsistent changes in different aspects of cervicovaginal tissue integrity. For example, the excipient-induced increase in paracellular permeability did not necessarily coincide with a decrease in TEER. Also, disodium EDTA did not change TEER at any of the concentrations tested; however, it caused a concentration-dependent increase in the mannitol permeability. In addition, the glycerin, propylene glycol, and methylparaben significantly increased mannitol permeability, which was disproportional to the moderate decrease in TEER. These observations were unexpected, since the decrease in TEER was often accompanied by an increase in permeability,^{36–38} due to the opening of the intercellular space by the co-incubated excipients. The transcellular permeability across the plasma membrane (³H-propranolol as the probe) was not affected by excipient treatment (Table 3), suggesting that the unparalleled change in TEER and paracellular permeability was not due to the compensation from altered transcellular permeability. Previous studies using kidney cell cultures suggested that paracellular permeability to electrical currents and non-charged solutes may be regulated by different sets of tight junctional proteins.^{39–43} If this is the case, given that multiple types of tight junctional proteins are expressed in human cervicovaginal mucosa,^{9,44–46} it is possible that EDTA treatment affected certain tight junction proteins that control small-molecule movement, but it did not affect those that control electrical current flux. Such a change in the organization of tight junction, in our opinion, is not comparable to the physical breaches that facilitate HIV-1 entry into the submucosal compartment,^{47–49} due to the difference in size and the entry mechanism between HIV-1 and mannitol. Both cell-free and cell-associated HIV-1 viruses are large, densely charged particles (>100 nm in diameter), and their entry process depends on interactions between virus and host cells.^{50,51} Conversely, mannitol is a small, neutral

molecule with a few nm diameter; its tissue penetration does not require interaction with cells; and it is much simpler than HIV-1 particles. Therefore, the excipient-induced increases in paracellular permeability do not necessarily predict increased risk of HIV-1 entry. From this perspective, the permeability assay alone cannot accurately reflect the comprehensive effect of treatments on the tissue defense barrier against pathogens. Rather, it should be used in combination with other methods (e.g., TEER, histology) for tissue integrity assessments.

Although several excipients altered mannitol permeability through the human ectocervical epithelia at tested concentrations, no obvious changes on tissue morphology or TEER were observed. This was also confirmed in the gel TEER studies in which neither vaginal TFV gel nor HEC gel impacted the TEER of ectocervical epithelium over the tested time period. We have published data showing that exposure to diluted TFV vaginal gel resulted in epithelial fracture in three of five ectocervical explant tissues without significantly decreasing tissue viability.⁵ The TFV (1%) and HEC gel tested in the study used the same formulation that was used for several microbicide clinical trials such as CAPRISA004. Both of the HEC and TFV gel formulations contained hydroxyethylcellulose, glycerin, EDTA, citric acid, methylparaben, and propylparaben. The *ex vivo* efficacy study was performed to test whether the altered permeability would impact tissue infectivity or TFV's anti-HIV effectiveness. The results show that the excipients at tested levels did not affect TFV effectiveness, and they indicate that there is no definite correlation between the altered paracellular permeability and TFV efficacy. Based on our results, a several-fold increase in mannitol permeability appeared insufficient to significantly facilitate the entry of HIV particles (Table 2 and Fig. 5).

Carias *et al.* studied the interaction of HIV with the mucosal barriers of the female reproductive tract and found that EDTA (10 mM) could increase virus penetration in human cervical explants.⁵² Several experimental differences between the study by Carias *et al.* and this study can explain this seemingly contradictory result. The cultured tissues in the study by Carias *et al.* are different from the human tissue culture used in our study in terms of the susceptibility to HIV infection. In Carias' paper, nonpolarized explants were used and were exposed to EDTA for 2 h, followed by exposure to HIV-1_{BaL} virus for 4 h. However, in our study, polarized explants were used and were exposed to HIV virus for 24 h. In the setting of a non-polarized culture (Carias *et al.*), both epithelium and stroma were exposed to the treatment and virus. In the polarized explant culture (our study), only the epithelium was exposed to the treatment and virus, which, in our opinion, more closely mimicked the *in vivo* scenario of HIV sexual transmission. Also, the EDTA concentration used in the study by Carias *et al.* (10 mM) is different from the concentration used in our study, and it may not be relevant to the *in vivo* EDTA concentration after the use of the marketed products. In addition, an increase in virus penetration does not necessarily equate to increased infection in the tissue, and it is not certain whether this increase in virus penetration is severe enough to cause a clinical impact. In our study, we examined the viral infection marker (p24 concentration), which is not the same as virus penetration.

Due to the limited availability of human tissues, both fresh and freeze-thawed human ectocervical tissues were used in this study. Our laboratory had previously evaluated the effect of the freeze-thaw process on tritiated water permeability through human cervicovaginal tissue, and no statistically significant differences in permeability values between fresh and freeze-thawed tissues were observed. We have also compared the $P_{\text{app-man}}$ between fresh and freeze-thawed human tissues used in this study; the ^{14}C -mannitol apparent permeability for fresh ($n=4$) and freeze-thawed ($n=4$) tissue was 0.352 ± 0.190 and 0.492 ± 0.286 cm/s, respectively. The mannitol permeability values were not significantly different ($p = .188$) between the two kinds of tissues, indicating that all tissues used in this study had similar baseline permeability. In addition to the mannitol permeability, the MTT-based cell viability values of several excipients also showed an unparalleled change as compared with TEER/morphology/permeability results. In this study, benzoic acid (0.15%) and sorbic acid (0.125%) caused unexpectedly significant MTT decreases (Table 2), in contrast to their TEER, histology, and mannitol permeability data, all of which appeared unchanged after incubation with these two excipients. The effect of these excipients on MTT values was probably due to their reported ability to interfere with mitochondrial activity instead of destructive cytotoxicity. The MTT assay is known to give false-positive or false-negative results on the cytotoxicity of tested agents,^{53–55} which is related to the intrinsic limitation of this assay. The agents that do not kill cells but inhibit metabolism may cause the MTT value to decrease as if the treatment could damage the tissue. On the other hand, the cytotoxic effect could be masked if the tested agent could cause moderate cell death but metabolically activate the remaining live cells. In this scenario, the net MTT value may appear unchanged. Therefore, the MTT assay was reliable in reflecting epithelial integrity only when the epithelium was severely damaged, after which most live cells were lost. Indeed, our data showed that MTT results correlated well with TEER and histology findings only when the excipient treatment greatly destroyed the epithelial cells. Therefore, this assay can be used to validate the devastating observations from TEER or histology measurements, but it cannot be used as the sole approach to examine epithelial integrity.

Due to the limited information regarding the tissue donor, we did not consider the donors' menstrual cycle stage or hormone use in the current study. However, besides the consideration on formulation, other biological factors such as use of hormonal contraceptives and genital tract inflammation have been reported to result in changes in vaginal barrier function and immune cell composition, which may increase susceptibility to HIV infection. Progesterone-based contraceptives, such as medroxyprogesterone acetate (MPA) injectable contraceptives, are commonly used by women in sub-Saharan Africa, where the HIV risk is high. Mitchell *et al.* investigated the effect of MPA on lymphocyte compositions in the female reproductive tract. They found that MPA significantly decreased median CD4+ and CD3+ T cell count in vaginal mucosal tissue 12 months after the MPA injection.⁵⁶ On the other hand, inflammatory cytokines, such as interleukin-8, in the macaque genital tract were found to be essential for establishment of a vaginal simian immunodeficiency virus infection model.⁵⁷ In humans, increased levels of inflammatory cytokines were observed in the genital tract

of those with a greater susceptibility to HIV. Masson *et al.* investigated whether genital inflammation impacts HIV acquisition in young women with high HIV incidence rates in Africa and found that a genital inflammatory profile contributed to a high risk of HIV acquisition.⁵⁸ STIs that cause inflammation, such as HSV-2 infection, cervicitis, urethritis, and vaginosis, are associated with increased susceptibility to HIV infection.^{59–63} Although these hormone-related epithelial changes are beyond the scope of this study, they are worth further investigation.

Due to the limited tissue availability and difficulties in mimicking the *in vivo* exposure scenarios, there are some limitations in the current study. Each excipient was tested by using tissues from three to five donors. Therefore, statistical analysis between donors could not be performed. Also, for most of the excipients tested, only one concentration was evaluated, which represented a standard level commonly used in pharmaceutical formulations. This concentration may have caused over- or under-estimation of the effect of excipients on tissue integrity. Therefore, additional studies are warranted to test whether any findings are concentration dependent. Biologically, the excipient concentration in vaginal microbicide gel will be diluted *in vivo* by genital tract fluid and mucus; therefore, the effect of excipients observed *in vitro* may directly mimic *in vivo* conditions. Future work is also needed to investigate the mechanism of the increased $P_{\text{app-mannitol}}$ after exposure to the excipients.

Conclusion

In summary, this study has examined the effect of generally-regarded-as-safe excipients on cervicovaginal tissue integrity, and it has provided implications for future development of vaginal PrEP products. Although clinical use of drug products containing these excipients has not been linked with severe side effects, our study has revealed that a number of excipients could alter certain aspects of cervicovaginal tissue integrity, in a concentration- and/or time-dependent manner. Although severe side effects are not reported regarding the clinical use of products containing these excipients, caution should be implemented when using these excipients in the context of HIV-1 prevention. Future investigations are warranted to identify factors that can lead to increased tissue exposure to excipients, to reduce the toxicity risk associated with excipients and to optimize clinical product use. In addition, our study has demonstrated that TEER, morphology, permeability, and MTT-based tissue viability do not necessarily change in parallel with each other. The use of a single measurement cannot accurately reflect the effect of excipients on cervicovaginal tissue, and it appears necessary to use a combination of multiple approaches to gain a comprehensive understanding. Further, we found that the treatment with EDTA, methylparaben, and propylene glycol at tested levels had no effect on the efficacy of TFV in preventing HIV-1 infection in an *ex vivo* explant tissue model. Taken together, the information generated in this study and future investigations will facilitate the efforts toward optimized vaginal PrEP products for HIV-1 prevention.

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Authors' Contributions

Conceived and designed the experiments: L.C.R., C.S.D., and M.H. Performed the experiments: M.H. and T.Z. Analyzed the data: M.H. and T.Z. Wrote the article: M.H., T.Z., C.S.D., and L.C.R.

Author Disclosure Statement

No competing financial interests exist.

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