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In vitro and *in vivo* evaluation of two carrageenan-based formulations to prevent HPV acquisition

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ABSTRACT

Commercial vaccines against human papillomavirus (HPV) have low uptake due to parental autonomy, dosing regimen, cost, and cold chain storage requirements. Carrageenan (CG)-based formulations prevent HPV infection *in vitro* and *in vivo* but data are needed on the durability of anti-HPV activity and the effect of seminal plasma (SP).

The Population Council's PC-515 gel and the lubricant Divine 9 were tested for their physicochemical properties and anti-HPV activity against HPV16, 18, and 45 pseudoviruses (PsVs). Anti-PsV activity was estimated using the luciferase assay in HeLa cells and the HPV PsV luciferase mouse model. Formulations were applied intravaginally either 2 h pre/2 h post (−2 h/+2 h) or 24 h pre (−24 h) relative to challenge with HPV16 or 45 PsV in PBS or SP/PBS.

Both formulations showed broad-spectrum anti-HPV activity *in vitro* (IC_{50}: 1–20 ng/ml), significantly decreasing HPV PsV infection in the mouse model (−2 h/+2 h, $p < 0.0001$). PC-515 protected better than Divine 9 in the −24 h dosing regimen ($p < 0.0001$) and comparable to Divine 9 in the −2 h/+2 h regimen ($p = 0.9841$). PC-515 retained full activity in the murine model when PsV solutions contained human SP. The durable, potential broad-spectrum anti-HPV activity of CG formulations in the presence of SP supports their further development to prevent HPV acquisition.

1. Introduction

HPV is the most common sexually transmitted infection (STI). Forty different HPV types infect the anogenital mucosa; 15 have been associated with carcinogenesis. HPV-16 and 18 account for most of the invasive cervical and anal cancers, although co-infection with other carcinogenic genotypes occurs (Armstrong, 2010; Massad et al., 2009; Schiller et al., 2012).

Gardasil®, a quadrivalent vaccine (types 6, 11, 16 and 18), and Cervarix®, a bivalent vaccine (types 16 and 18), prevent new HPV infections (Schiller et al., 2012). Both are listed as subsidized vaccines in underserved and poor countries by Global Alliance for Vaccines and Immunization. The benefits of a global HPV vaccination program are undeniable. But the vaccines do not protect against 36 other HPV types associated with anogenital infections and have low uptake due, in part, to high cost and cold chain storage requirements (Lowy and Schiller, 2012).

Results from the CAPRISA-004 trial of vaginally administered tenofovir gel (Abdool Karim et al., 2010) and oral pre-exposure prophylaxis (PrEP) trials (Celum and Baeten, 2012) showed that oral or topical PrEP can prevent STI acquisition. Carrageenan (CG), a seaweed-derived polysaccharide, potently inhibits HPV infection *in vitro* (at neutral and acidic pH) and *in vivo* (Buck et al., 2006; Roberts et al., 2007). Additionally, analysis of data from highly adherent participants in the Carraguard (PC-515, 3% CG) Phase 3 trial suggested that CG decreases HPV acquisition (Marais et al., 2011). These data, combined with the excellent safety profile of CG (Crostarosa et al., 2009; Kilmarx et al., 2008, 2006; Martin et al., 2010; Skoler-Karpoff et al., 2008; Turville et al., 2008; Whitehead et al., 2006), have supported clinical testing of PC-515 and Divine 9 gel to prevent HPV. Here we compare the physicochemical properties of both gels, examining their *in vitro* and *in vivo* efficacy against different HPV types. Additionally we evaluate the effect of SP on the *in vivo* anti-HPV activity of PC-515.

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2. Materials and methods

2.1. Gel preparation

High lambda CG (Gelymar, Puerto Montt, Chile) was dissolved at 3% (w/v) in phosphate buffered saline (PBS) at 70 °C, 3 h, 40 rpm in a DPM 3 Mixer (Charles Ross and Son Company, Hauppauge, NY). Methylparaben (Spectrum Chemical, New Brunswick, NJ) in PBS was added (0.2% final concentration) and the solution stirred for 1 h at 40 rpm. The pH was adjusted to 6.5–7.0 with 1 N HCl (Ricca Chemical, Pocomoke City, MD). Bubbles were removed by stirring for 15 min under vacuum. Clean Chemical Sweden (Borlänge, Sweden) manufactured hydroxyethylcellulose (HEC) placebo gel, using the literature procedure (Tien et al., 2005). Non-sulfated cellulose derivatives like HEC are inactive against HPV (Buck et al., 2006). HEC gel is the universal microbicide placebo, having substantial safety data (Richardson et al., 2013).

2.2. Gel properties and CG content

PC-515 and Divine 9 (Divine Corporation, Orlando, FL) were tested for viscosity, rheology, pH, osmolality, turbidity, and CG content. Viscosity was measured using a calibrated Brookfield (Middleboro, MA) DV-II + viscometer (SC4-28 spindle, 5 rpm, SC4-13RPY chamber, 37 °C). Rheology was characterized using a calibrated AR 1500ex Rheometer (TA Instruments, New Castle, DE) outfitted with 4°, 40 mm diameter, and 108 μm truncation geometry. Viscosity was measured over shear rates of 0.1–120 s^{-1}. Gel pH was tested using an Orion 4 Star digital pH Meter (ThermoFisher Scientific, Waltham, MA). Osmolality was measured using a calibrated Vapro 5520 osmometer (Wescor, Logan, UT). Formulations were considered iso-osmolal or nearly iso-osmolal at 200–500 mOsmol/kg. Turbidity was measured using the absorbance (vs. standards) of a sample at 450 nm in an Emax plate reader (Molecular Devices, Sunnyvale, CA). CG content was determined using methylene blue (Soedjak, 1994).

2.3. Cells and viruses

HeLa cells (ATCC, Rockville, MD) were grown in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Life Technologies) and 50 U/ml of penicillin and 50 μg/ml streptomycin (Life Technologies).

HPV-PsVs: HPV16, 18, and 45 PsVs were produced as previously described (Kizima et al., 2014). HPV-PsV stocks were titered by quantitative PCR (qPCR), in an ABI ViiA 7 thermal cycler (Kizima et al., 2014).

2.4. Cytotoxicity and anti-HPV activity

Cytotoxicity and anti-HPV activity were tested in HeLa cells (Kizima et al., 2014). Briefly, HeLa cells were plated (10^4 cells/well) in 100 μl of medium and incubated overnight at 37 °C, 5% CO_2, and 98% humidity (standard conditions). Test gels were diluted in medium to obtain 2× dilutions of the appropriate dilution range. Cell culture media on the cell monolayers was replaced with 50 μl of the diluted formulations or 50–100 μl of medium for virus and cell controls. Dilutions were tested in triplicate. Fifty μl of HPV 16, 18 or 45 PsVs (5×10^5 copies) were added to all wells with the exception of cell controls and incubated for 72 h at standard conditions. Cells were lysed to detect luciferase activity using the Pierce Firefly Luciferase Glow Assay with Pierce Firefly Signal Enhancer (Thermo Scientific) as described by the manufacturer. Luminescence was read on a Gemini EM microplate reader (emission 542 nm) using Softmax Pro 3.2.1 software. Cytotoxicity was estimated using the XTT assay (Fernández-Romero et al., 2007), mimicking the antiviral assay but without virus. CC_{50} and IC_{50} values were calculated using a dose–response–inhibition analysis on GraphPad Prism v5.0c software. Therapeutic indexes ($TI = CC_{50}/IC_{50}$) were calculated.

2.5. HPV-16 and 45 PsV mouse model

We followed the Animal Welfare Act (Code of Federal Regulations, 2001) and the Guide for the Care and Use of laboratory Animals (National Research Council, 2010). Rockefeller University's Institutional Animal Care and Use committee (IACUC) of the Comparative Bioscience Center (CBC) approved animal protocols. We tested the *in vivo* anti-HPV activity of CG formulations using the mouse HPV PsV model (Kizima et al., 2014; Roberts et al., 2007). Ten μl of PC-515, Divine 9, or HEC were applied intravaginally at 24 h, 2 h, or 10 min before challenging with 8×10^6 copies/10 μl of HPV16 PsV. Separately, we also applied PC-515 or HEC gel −2 h/+2 h virus challenge with HPV16 or HPV45 PsV in the presence or absence of 100% pooled human SP (Lee Biosolutions, St. Louis, MO).

2.6. CG pharmacokinetics (PK) in mice and CG detection

PK studies were performed by instilling intravaginally 10 μl of PC-515 or Divine 9 ($n = 6$ per gel). Vaginal washes (200 μl of D-PBS) were collected after 1, 2, 4, 8 or 24 h. Native cervicovaginal fluid volume was not factored into the final calculations. A CG ELISA was used to quantify CG [Lower Limit of Quantification = 40 ng/ml] (Kizima et al., 2014).

2.7. Statistical analyses

ANOVA was used to analyze the log-transformed radiance across treatments in the HPV PsV mouse model. The F test was used for overall comparison between treatments and pairwise comparisons were performed using Tukey–Kramer adjusted *t* tests. Areas under the curve between 1 and 24 h ($AUCs_{1-24}$) were compared using a t test with pooled variance on the natural logarithm of these AUCs.

3. Results

3.1. Carrageenan content and gel properties

PC-515 contains more CG than Divine 9: 30.6 mg/ml and 14.0 mg/ml, respectively, (Table 1). Divine 9 is less viscous than PC-515 and hypo-osmolal; PC-515 is iso-osmolal. Both are shear-thinning gels, becoming less viscous at shear stresses (Fig. 1) experienced during sexual activity. PC-515 has the higher initial, terminal, and overall maximum viscosities.

3.2. In vitro and in vivo efficacy against HPV PsVs

PC-515 and Divine 9 were active *in vitro* against three predominant HPV types (16, 18 and 45) (Fig. 2). Their IC50 values were between $1/10^6$ and $1/10^7$ based on gel dilutions, which corresponds to CG concentrations between 1 and 20 ng/ml. Neither

Table 1
Formulation attributes.

Property	PC-515	Divine 9
Carrageenan content (mg/ml)	30.6	14.0
pH	7.0	7.0
Osmolality (mOsmol/kg)	326	<100
Viscosity (cP @ 37 °C, 5 rpm)	33,000	1600

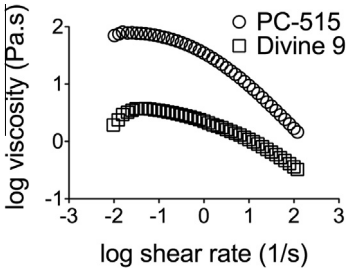

Fig. 1. Rheological profiles of PC-515 and Divine 9 demonstrate differences in magnitude of viscosity, but similar shear thinning behavior. Rheological samples were analyzed in a shear rate flow from 0.1 to 120 s^{-1}, while monitoring viscosity. The graph shows the log viscosity vs. log shear rate.

diluted formulation was toxic in HeLa cells (Fig. 2A; CC_{50} > 0.016). Both showed highly selective (TI above 17,000) antiviral activity (Fig. 2B).

In vivo efficacy was determined by applying formulations 24 h pre challenge or 2 h pre/2 h post to mimic the CAPRISA 004 dosing regimen and that proposed for a Phase 2b clinical trial at Albert Einstein College of Medicine (AECOM). Regardless of dosing regimen, PC-515 and Divine 9 significantly decreased HPV16-PsV infection compared to HEC placebo ($p < 0.0001$ vs. PC-515 or Divine 9) (Fig. 3A and Table 2). PC-515 protected significantly better than Divine 9 when applied 24 h before PsV challenge ($p < 0.0001$), protecting as effectively as Divine 9 applied 2 h pre/2 h post PsV challenge ($p = 0.9841$) (Fig. 3A and Table 2).

In a separate experiment, CG was detected in mouse vaginal washes 1, 2 and 4 h post-PC-515 application at concentrations between 50 and 200 μg/ml, which dropped off at 24 h to between 20 and 80 μg/ml (Fig. 3B). CG concentrations were lower in the Divine 9 washes taken at the same times; no CG was detected at 24 h. The CG $AUC_{1-24\ h}$ in the mice receiving PC-515 is 9.3 times higher than for mice receiving Divine 9 (p-value < 0.0001). CG concentrations after PC-515 dosing are 3000–10,000 times CG's *in vitro* IC_{50} (see Table 3).

Knowing that women could be exposed to HPV admixed with SP and several sulfated/sulfonated polymers demonstrated reduced antiviral activity in the presence of SP or in postcoital sampling (Herold et al., 2011; Keller et al., 2010; Patel et al., 2007; Segarra et al., 2011), we tested the antiviral activity of CG against HPV-16 and HPV-45 PsV in the presence of SP. These main oncogenic HPVs rendered high enough titers to use in the HPV PsV mouse model. Fig. 4 shows that PC-515 retained potent anti-HPV16 and 45 activities when applied 2 h pre/2 h post PsV challenge in the presence or absence of 100% pooled human SP compared to placebo-treated mice ($p < 0.0001$).

4. Discussion

Complementary strategies to decrease HPV infections include combining topical pre or post exposure microbicides and vaccines for primary prevention with early detection and treatment of infection by carcinogenic HPV types.

We previously reported the anti-HPV activity of an MZC gel containing 3% CG (Kizima et al., 2014). Here we examine CG-only formulations that are being considered for Phase 2b trials, looking at their *in vitro* and *in vivo* efficacy against HPV and the durability of the activity. MZC gel's anti-HPV activity is due entirely to its CG content, so we do not expect any difference in the anti-HPV activity of MZC gel and PC-515.

CG's *in vitro* activity is independent of the CG type (lambda, kappa, iota) and might be significant when applied after virus challenge (Buck et al., 2006). CG is thought to inhibit HPV binding to heparan sulfate on cell surface proteoglycans, but may also block subsequent events that facilitate virion binding and entry into epithelial basal cells (Buck et al., 2006). We report inhibition of PsVs produced in 293T cells. These PsVs may have a different mode of adsorption compare to HPVs produced in raft cultures (Cruz and Meyers, 2013). However, CG's ability to block post-adsorption steps may afford similar inhibition using both virus sources.

CG's anti-HPV IC_{50} ranges from 1 to 20 ng/ml; CG's anti-HIV IC_{50} is approximately 3 μg/ml (Fernández-Romero et al., 2007). This 1000-fold difference could explain the lack of anti-HIV activity in the Phase 3 Carraguard trial but the potential anti-HPV activity in a subgroup of adherent women in the trial (Marais et al., 2011). The weak anti-HIV activity and short duration of first generation microbicides (mostly polyanions), plus low gel adherence, may partially explain the results of the first generation clinical trials (Romano et al., 2012). Here we demonstrated a significant

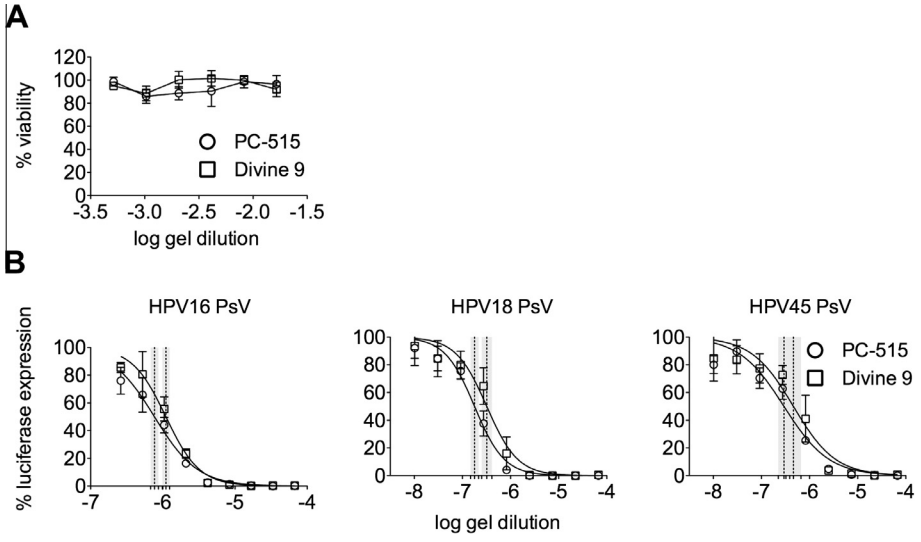

Fig. 2. PC-515 and Divine 9 are non-toxic and have a potent and broad anti-HPV activity *in vitro*. (A) Cytotoxicity was estimated using the XTT assay in HeLa cells. (B) The anti-HPV-16, 18 and 45 IC_{50} values (shown as a vertical dotted line within the 95% confidence interval shaded in gray) were estimated using the luciferase assay in HeLa cells. All gel dilutions were tested in triplicate showing mean ± SD. Therapeutic Indexes (TI = CC_{50}/IC_{50}) are >17,000 for both formulations.

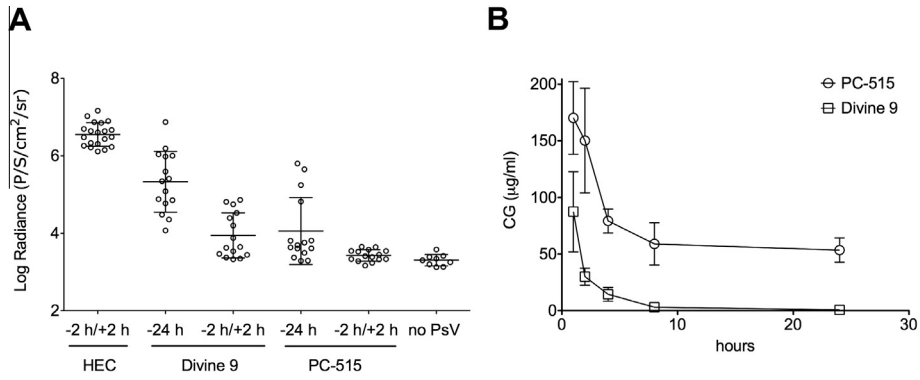

Fig. 3. PC-515 is significantly more protective to mice against vaginal HPV-16 PsV infection than Divine 9. (A) Depo-treated Balb/C mice were given the indicated formulations (HEC, PC-515, or Divine 9) intravaginally at 24 h before (−24 h) or 2 h before and 2 h after (−2 h/+2 h) HPV 16 PsV challenge (n = 15/treatment). *In vivo* luciferase expression is expressed as mean luminescence in photons per second per centimeter square per steradian ± SD for each individual animal. The statistical analysis comparing treatment groups is shown in Table 2. (B) CG levels (mean µg/ml ± SD) in vaginal washes from mice treated intravaginally with PC-515 or Divine 9 were determined at 1, 2, 4, 8 and 24 h post-gel (n = 6 per time point). There is significant difference ($p < 0.0001$) between PC-515 AUC_{1-24} (geometric mean AUC_{1-24} h·µg/ml = 1426.3) and Divine 9 AUC_{1-24} (geometric mean AUC_{1-24} h µg/ml = 153.8). CG concentrations were assumed to be log-normally distributed and a LLOQ of 40 ng/ml was taken into consideration.

Table 2
Statistical analysis comparing PC-515 vs. Divine 9 in the HPV mouse model.

Pairwise comparison				Tukey–Kramer (p value)
Formulation 1	Treatment relative to HPV challenge	Formulation 2	Treatment relative to HPV challenge	
Divine 9	−24 h	HEC	−2 h/+2 h	<0.0001
PC-515	−24 h	HEC	−2 h/+2 h	<0.0001
Divine 9	−2 h/+2 h	HEC	−2 h/+2 h	<0.0001
PC-515	−2 h/+2 h	HEC	−2 h/+2 h	<0.0001
PC-515	−24 h	Divine 9	−24 h	<0.0001
Divine 9	−2 h/+2 h	Divine 9	−24 h	<0.0001
PC-515	−2 h/+2 h	Divine 9	−24 h	<0.0001
Divine 9	−2 h/+2 h	PC-515	−24 h	0.9841
PC-515	−2 h/+2 h	PC-515	−24 h	0.0338
PC-515	−2 h/+2 h	Divine 9	−2 h/+2 h	0.1220

Table 3
In vivo PC-515 efficacy in the presence or absence of SP.

Pairwise comparison				Tukey–Kramer (p value)
Formulation 1	HPV inoculum media	Formulation 2	HPV inoculum media	
HPV 16 PsV				
HEC	PBS	HEC	SP	0.1398
PC-515	PBS	HEC	PBS	<0.0001
PC-515	SP	HEC	PBS	<0.0001
PC-515	PBS	HEC	SP	<0.0001
PC-515	SP	HEC	SP	<0.0001
PC-515	SP	PC-515	PBS	0.9704
HPV 45 PsV				
HEC	PBS	HEC	SP	0.0051
PC-515	PBS	HEC	PBS	<0.0001
PC-515	SP	HEC	PBS	<0.0001
PC-515	PBS	HEC	SP	<0.0001
PC-515	SP	HEC	SP	<0.0001
PC-515	SP	PC-515	PBS	0.9996

reduction in HPV infection even when applying PC-515 24 h before HPV PsV challenge.

The complete reduction of HPV PsV infection by PC-515 (−2 h/+2 h) is associated with CG levels of around 100 µg/ml in vaginal washes; Divine 9 produces similar levels for only 1 h after single gel application. Similar CG concentrations (median: 98 µg/ml, range, <25–282 µg/ml) have been found in a Phase 1 safety trial where cervicovaginal lavages (CVL) were collected 8–24 h after vaginal application of a 3% CG gel (Haaland et al., 2012). These results suggest that PC-515 will durably protect adherent participants.

Our *in vivo* data show that CG's anti-HPV activity is unaffected by the presence of SP. SP was shown to inhibit the binding of PRO 2000 and cellulose sulfate to HSV-2, resulting in decreased protection of mice intravaginally challenged with HSV-2 (Patel et al., 2007). Additionally, low postcoital bioavailability and antiviral activity of PRO 2000 in CVLs support the experiments done in mice (Keller et al., 2010). Cellulose sulfate showed decreased antiviral activity in the presence of SP and enhanced viral replication probably by disrupting the epithelial barrier (Mesquita et al., 2009). These results may explain why PRO 2000 and cellulose sulfate failed to prevent HIV or HSV-2 in clinical trials.

PC-515 and Divine 9 have similar *in vitro* IC_{50} values when the formulations are diluted equally. However, PC-515 protects better against HPV PsV infection *in vivo* compared to Divine 9, which is perhaps due to differences in their physicochemical and rheological properties.

Here we show that formulations with similar *in vitro* efficacy but different rheological properties have significantly different PK profiles and *in vivo anti*-HPV activity. Shear thinning is a critical gel property, ensuring that during sexual activity the gel will spread uniformly throughout the vagina, protecting all surfaces against viral infection. A computational model was used to determine the optimal rheology for intravaginal gel spreading (Fernández-Romero et al., 2012), showing that PC-515 and other thixotropic CG gels with viscosities between 24,000 and 40,000 cP cover the entire surface area of the human vagina (100 cm^2). Lower viscosity gels like Divine 9 may spread effectively. However, they may leak or be washed away rapidly, result-

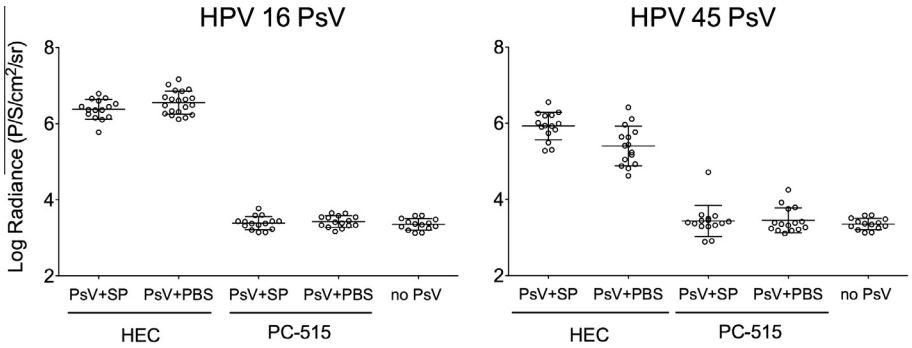

Fig. 4. Seminal plasma does not affect the *in vivo* anti-HPV activity of PC-515. Depo-treated Balb/C mice were given the indicated formulations (HEC or PC-515) under the −2 h/+2 h regimen relative to challenge with either HPV 16 or HPV 45 PsV challenge in SP or PBS (n = 15/group). *In vivo* luciferase is expressed as mean luminescence in photons per second per centimeter squared per steradian ± SD for each individual animal. The statistical analysis comparing treatment groups is shown in Table 3.

ing in sub-effective concentrations of gel in the vaginal lumen, shorter durability, and poor acceptability.

Divine 9 is hypo-osmolal; PC-515 is nearly iso-osmolal. Hyper-osmolal or hypo-osmolal vaginal or rectal formulations may damage epithelial tissue, leading to increased susceptibility to STIs. We and others have investigated the effect of osmolality of sexual lubricants on the integrity of the epithelium, finding that a number of hyper-osmolal commercial lubricants are associated with cellular toxicity and epithelial damage (Begay et al., 2011; Dezzutti et al., 2012; Fuchs et al., 2007). Moreover, a recent publication suggests that some lubricants may increase vulnerability to STIs (Gorbach et al., 2012). Although concerns have been raised primarily around hyper-osmolal formulations, the safety of a hypo-osmolal formulation like Divine 9 should be investigated and addressed, if needed.

CG was the main ingredient in Carraguard, an experimental microbicide gel that the Council tested in a Phase 3 clinical trial (Skoler-Karpoff et al., 2008). CG gel does not damage vaginal or rectal epithelial tissue in mice. It does not increase susceptibility to HSV-2 vaginal infection in mice and is nontoxic to *Lactobacillus jensenii* or *Lactobacillus crispatus in vitro* (Kenney et al., 2013). Experiments in macaques show that repeated application is safe with no effects on vaginal pH or cytokines and chemokine levels in fluids (Kenney et al., 2011). Finally Phase 1, 2 and 3 clinical trials have demonstrated that vaginal application of a 3% CG gel is safe and acceptable (Kilmarx et al., 2008, 2006; Martin et al., 2010; Skoler-Karpoff et al., 2008; Whitehead et al., 2006).

Considering the excellent safety profile, rheological properties, and anti-HPV activity of a 3% CG gel, we have used CG-based gels as delivery vehicles for anti-HIV and anti-HSV-2 APIs (Fernández-Romero et al., 2012; Kenney et al., 2011, 2013, 2012; Kizima et al., 2014).

A strategy combining microbicides like PC-515 and MZC with vaccination and early diagnosis could effectively prevent HPV acquisition. CG significantly prevents *in vitro* and *in vivo* infection by different HPV genotypes (Buck et al., 2006), overcoming one of the limitations of the current vaccines. However, the recent VOICE Trial results showed that microbicide gels face significant adherence challenges (Van Damme et al., 2012; van der Straten et al., 2012), some of which are related to fear of partner's detection of gel use and uncooperative partners (Muchomba et al., 2012). Adherence issues may also be linked to daily-use products, since potential users may not acknowledge their HPV, HSV-2, or HIV risk daily. These issues prompted the Council and others to develop delivery systems like intravaginal rings and nanofibers to increase adherence.

Our results confirm the durable, broad-spectrum *in vivo* activity of a CG gel against HPV, even in the presence of SP. These results support further testing of CG formulations to prevent HPV acquisition.

Conflict of interest

All authors agree that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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