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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.

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Additionally we evaluate the effect of SP on the *in vivo* anti-HPV activity of PC-515.

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.

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

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