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MATRIX-BASED CONTROLLED RELEASE DELIVERY OF ACYCLOVIR FROM POLY-(ETHYLENE CO-VINYL ACETATE) RINGS

Nicholas J. Giannasca^{1,§}, Jennifer S. Suon^{1,§}, Amanda C. Evans¹, Barry J. Margulies^{1,2,3,*}

¹Towson University Herpes Virus Lab, Towson University Department of Biological Sciences, Towson MD

²Molecular Biology, Biochemistry, and Bioinformatics Program, Towson University, Towson MD

³Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore MD

Abstract

Up to 85% of the US adult population carries herpes simplex virus type-1 (HSV-1), with a smaller percentage (22%) infected with HSV-2. Herpesviruses can survive in lytic phase, when the viruses are actively replicating, or in latency, when the virus is functionally dormant in ganglia. Among drugs to treat these infections is acyclovir (ACV). ACV exhibits poor oral bioavailability and a short in vivo half-life; only about 10–15% of ingested drug enters the bloodstream and its half-life is about 3 hours. With those disadvantages and the possibility of poor patient compliance, viral replication may not always be suppressed. To abrogate these shortcomings we propose local distribution via sustained drug release. We present a matrix-based antiherpetic ring, composed of poly(ethylene co-vinyl acetate), that releases ACV directly to the vaginal epithelium. A 30-day in vitro drug release trial showed that approximately 135 $\pm$ 20 μ g/day of ACV was consistently released. Rings were nontoxic in cell culture and suppressed primary HSV-1 and HSV-2 replication. We expect these data form the basis for novel interventions in human health, where new prophylactics and therapeutics against genital herpes are truly needed.

Graphical Abstract

*To whom correspondence should be addressed. Department of Biological Sciences, Towson University, 8000 York Rd., Towson, MD 21252. Phone: (410)-704-5019. FAX: (410)-704-2405, bjrmarg@alum.mit.edu.

§Equal contributors

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Keywords

Herpes; genital herpes; acyclovir; poly(ethylene-co-vinyl acetate); vaginal ring; controlled release

Introduction

Herpes simplex viruses-1 and -2 (HSV-1 and HSV-2) cause primary and recurrent genital infections in humans [1]. The most common treatment prescribed is oral administration of acyclovir (ACV) or its more readily absorbed prodrug, valacyclovir [2]. ACV is used to treat those infected by HSV-1 and HSV-2, but due to poor patient compliance, poor oral bioavailability [2–4], and short in vivo half-life [5, 6], oral dosing often shows inefficient viral suppression [2]. One complication arises when patients deem themselves too busy, or they forget to take their medications as prescribed, resulting in drug troughs. Oral dosing itself results in ill-absorption by the gut (about 10–20% of the original dose), resulting in lower efficacy and the need for larger doses of drug to compensate [2, 4]. Furthermore, ACV has a plasma elimination half-life of 2.5–3.3 hours, resulting in its plasma concentration dropping rapidly even after oral dosing [2, 4]. These three problems may result in inefficient viral suppression.

We show here a model antiherpetic ring that reduces these disadvantages associated with oral dosing. While oral medication eventually delivers drug systemically, a ring would be

placed at the targeted site, releasing higher local concentrations of ACV directly to the vaginal epithelium where genital herpes symptoms appear; local drug distribution eliminates the bioavailability issues associated with the oral uptake, systemic travel, and renal metabolism of drug before it can reach its target tissue [2]. The device would allow women the convenience of a once-a-month intervention for maintenance of their anti-herpetic treatment. Throughout the duration of time, the ring would offer women prophylaxis of HSV and potentially, for those already affected, effective viral suppression and reduction of recurrent outbreaks.

Materials and Methods

Creation of ACV-silicone rings

Initially, we experimented with silicone as a drug release platform. Although silicone could easily be combined with ACV to create a homogenous mixture [7, 8], it was limited to a 55% (w:w) composition of ACV. The silicone elastomer used was NuSil MED-4050 (NuSil Silicone Technology, Carpinteria, CA), which exists as silicone parts A and B. Silicone part A and B were combined in a 1:1 ratio using a rolling press as described in [7]. The drug-polymer mix was created essentially as described in [8], except either 50% or 55% ACV (w:w) (Advanced Scientific, Ft. Lauderdale, FL) was used. The final mixture was extruded through a 0.88-mm die using a one-ton Arbor press (Harbor Freight Tools, Calabasas, CA) to create smooth “noodles,” essentially as described in [7]. The newly formed product was cut into 12-mm rods. The rods were placed on a glass Petri dish and the ends of the rods were adhered to each other with the help of forceps to create a 2.3-mm inner diameter torus. After formation, the rings were cured at room temperature for 7 days, then at 60 °C for 24 hours [7, 8]. Control silicone rings containing 0% ACV were made using the same methodology.

Development of ACV:EVA25 and ACV:EVA40 rings

Poly (ethylene co-vinyl acetate) (EVA), a copolymer of ethylene and various weight percentages of vinyl acetate, was defined thusly: poly (ethylene co-vinyl acetate) 75:25 (w:w) was termed “EVA25” and poly (ethylene co-vinyl acetate) 60:40 w:w was termed “EVA40.” Acyclovir, EVA25 polymer beads (product #437220, Sigma-Aldrich, St. Louis, MO) and 300 mL of chloroform (LabChem, Zelienople, PA) were stirred together for 24 hours at room temperature in a chemical fume hood at either 50%:50% (w:w) or 65%:35% (w:w) drug:polymer to allow all solvent to evaporate out of solution. We found that 65% ACV to 35% polymer is the maximum drug load for this drug:polymer mixture. The dried mixture of ACV and EVA was placed in a glass Petri dish at 130 °C for 30 min to soften, then extruded through a 0.88-mm diameter die with a one-ton Arbor press, as described above [7]. The mixture was repeatedly processed through the die to condense the mixture and eliminate residual air pockets. Once all air pockets were eliminated, the final extrusion resulted in a smooth “noodle” as above. The resulting “noodle” was cut into 12-mm rods, which were then placed into another glass Petri dish at 120 °C to soften and bind the ends of the rod together to form a torus with an approximately 2.3-mm inner diameter (Fig 1). The same method was used when creating rings with EVA40 polymer beads (product #340502,

Sigma-Aldrich). Controls for both EVA25 and EVA40 were made using the same method as well, but no drug was introduced during the solvation/evaporation process.

In vitro drug release

Three rings of each type (50% ACV (w:w) silicone, 50% ACV (w:w) EVA25, 50% ACV (w:w) EVA40, and their corresponding drug-free counterparts) were sterilized [9] and placed in separate wells of a 24-well plate. Vaginal fluid simulant (VFS) [10] was placed in each well, 1 mL per well, and the plate was incubated at 37 °C in a humidified atmosphere at 5% CO₂ for one day. The VFS was collected and replaced every day for 7 days. The experiment was also repeated as described for a longer duration of 30 days.

Quantification of ACV in samples

Acetonitrile (450 µL) was added to 50 µL sample (VFS, collected as above) to quantify drug in solution as previously described [7, 8]. Briefly, samples were run isocratically at 0.4 mL/min with 10% of 0.1% formic acid/90% acetonitrile for 5 minutes per sample on a Luna HILIC column (Phenomenex, Torrance, CA); ACV was detected by UV absorbance at 251 nm. Quantities of drug determined by HPLC were compared against a 15-point standard dilution curve as previously described [7, 8]; standards ranged from 1000 µg/mL to 5 µg/mL. Each day of drug release is shown relative to day zero, when the rings were first placed in VFS. This assay has a reliable lower level of quantitation of 5 µg/mL [7, 8].

In vitro safety and efficacy of rings

Vero cells [ATCC CCL-81] were seeded into 12-well plates at a density of 1.0×10^5 cells per well in complete α -MEM (α -modification of minimal essential media [Corning CellGro, Corning, NY]) with 10% FBS (Thermo Fisher Scientific, Waltham, MA) and grown at 37 °C and 5% CO₂ in a humidified atmosphere. After 24 hours, Transwell inserts (Corning Costar, Kennebunk, ME) were added to each well. Rings with and without ACV and an ACV solution at 50 µg/mL were added to their respective wells; each treatment was conducted in triplicate. After another 24 hours, the Transwells (with or without devices) were removed and placed in an empty 24-well plate, followed by the media from each well; all were mapped 1:1 from the original plate to this temporary one. The monolayer of cells was infected with 100 µL of either HSV-1 (KOS), HSV-1 (17), HSV-2 (MS), HSV-2 (G) (ATCC #VR-1493 #VR-1789, #VR-540, and #VR-734, respectively) in Dulbecco's phosphate buffered saline (PBS; Corning CellGro) at an MOI of approximately 1, or mock-infected with PBS. The plates were placed back into the incubator for one hour and were shaken every 10 minutes to ensure efficient infection of cells. After one hour, the virus/PBS solution was removed from the cells, and the respective media and Transwell inserts (with/without devices) were returned to their wells. Once sufficient cytopathic effect (CPE) was seen (about 30 hours later), the Transwells and devices were removed from the wells, 700 µL of medium from each well was removed and placed into a cryovial (Corning), and glycerol (Thermo Fisher Scientific) was added to 10% for storage at -80 °C until titering.

MTT viability assays were conducted as previously described [9] to determine safety in a separate set of non-infected (but treated) wells. Briefly, non-infected cells were washed with PBS, then exposed to 400 µL of a solution of thiazolyl blue tetrazolium bromide (0.5

mg/mL) for 30 minutes at 37 °C/5% CO₂. DMSO (800 µL) was added to each well of cells, and a portion (200 µL) of the final solution was read at 570 nm and 620 nm in a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA). Corresponding OD readings were corrected for background absorption and divided by the absorbance for untreated cells to arrive at percent viability.

The medium collected was titrated by plaque assay based on a protocol adapted from [11]. A 6-well plate was seeded at 4.0×10^6 cells per plate in complete α -MEM. The next day the medium was removed from the cells three wells at a time, and 400 µL of serially 10-fold diluted samples were added to each well. The plates were returned to the incubator for one hour and were shaken every 10 minutes. The diluted sample was then carefully aspirated from the well and 2 mL of medium containing 5% FBS and 0.5% (hydroxypropyl)-methylcellulose (Sigma-Aldrich) in untreated α -MEM was added to the well. After 3 days the methylcellulose overlay was removed, and the cells were stained with 1% crystal violet (Sigma-Aldrich) in 50% ethanol at 37 °C for 30 min. The crystal violet was washed with tap water until the runoff was clear, and the plate was allowed to air-dry overnight. Plaques were counted and used to determine the viral titer of the original sample.

Statistical analysis of viral titers

One-way analysis of variance (one-way ANOVA) and a Tukey post-hoc test were performed. Individual means and standard deviations were compared between groups of samples to consider statistical significance between means. A p value < 0.05 represented a difference in means that was statistically significant, whereas a p value > 0.05 represented a difference in means that was not statistically significant.

Results

In vitro drug release

Silicone and EVA were chosen as the main polymers for this experiment because they are approved for use by the Food and Drug Administration for other medical devices and are commercially available [12, 13]. We monitored daily drug release for 7 days to compare ACV release from silicone, EVA25, and EVA40 containing the same concentration of ACV (50% w:w drug:polymer) in VFS. EVA25 displayed the highest drug release compared to all other experimental groups (Fig 2). By the fourth day, silicone-based rings released undetectable amounts of drug at 50% drug load, too low to be therapeutic [14], so we chose to continue our further studies with EVA25 and EVA40 at their physical maximum drug loads of 65% ACV:35% polymer.

Using the same method, various drug-polymer combinations of EVA25 and EVA40 were tested along a 30-day period (Fig 3). Initially 65% ACV:35% EVA40 consistently released the most drug but after the 10th day, 65% ACV:35% EVA25 released more over that time period. By the 18th day, 65% ACV:35% EVA40 discontinued drug release while 65% ACV:35% EVA25 continued to release the most drug throughout the rest of the trial. We therefore chose to use 65% ACV:25% EVA25 devices for the rest of our work.

Safety and efficacy against primary HSV-1 and HSV-2 infection

Vero cells were treated with either EVA25 rings containing no drug, EVA25 rings with 65% ACV, an ACV solution at a final concentration of 50 µg/mL, or α -MEM containing neither drug nor EVA. After one day of treatment, cells were either mock infected or infected with HSV-1 (KOS), HSV-1 (17), HSV-2 (MS), or HSV-2 (G) at an MOI of 1. An MTT assay of mock-infected cells that were treated as described showed that our EVA25 rings containing ACV, the EVA control rings, and a 50 µg/mL solution of ACV all appear to be nontoxic to cells when compared to cells that were left untreated ($p=0.9$) (Fig 4). These data are consistent with the in vivo safety profiles of EVA [15–17] and ACV [4, 18], both of which are clinically well tolerated in people [2, 4, 14–17].

The rings also were shown to prevent primary infection by various herpes simplex viruses (Fig 5). Extracellular virus particles were measured by plaque assays [9, 11]. Cells treated with ACV-containing rings exhibited 1.9 to 3.4 log lower titers than cells which received no drug, levels that were comparable to samples treated with ACV alone at 50 µg/mL. Furthermore, these lower titers were significantly different from titers of virus from the untreated cells. A two-tailed T test revealed that treatment with 65% ACV (w:w) EVA25 rings resulted in a statistically significant decrease in virus titer compared to wells that were not treated (for HSV-1 (17), $p<0.01$; for HSV-1 (KOS), $p=0.05$; for HSV-2 (MS), $p<0.01$; for HSV-2 (G), $p<0.01$) or treated with EVA rings containing no ACV (for HSV-1 (17), $p<0.01$; for HSV-1 (KOS), $p<0.01$; for HSV-2 (MS), $p<0.01$; for HSV-2 (G), $p<0.01$), and the rings exhibited greater or equal suppression of viral replication compared to the 50 µg/mL ACV solution (for HSV-1 (17), $p=0.02$; for HSV-1 (KOS), $p<0.01$; for HSV-2 (MS), $p<0.01$; for HSV-2 (G), $p=0.15$). These data show the breadth of applicability of these devices against multiple strains of two different clinically relevant HSVs, demonstrating their potential for use against HSVs in vivo.

Discussion

Because HSV has such a high seroprevalence in the United States, a method of dosage that consistently and efficiently releases medication to prevent infection or help decrease recrudescence needs to be developed. We believe this is especially important because individuals with genital herpes are much more susceptible to HIV infection [19]. Therefore, treatment and prevention of genital herpes is a valuable method of protection against HIV infection.

Our experiments were looking for the best drug and polymer combinations that offer consistent and effective drug release over an extended period of time in order to effectively suppress herpes viruses through a more potent and longer lasting treatment. In vitro experiments showed those drug levels can be achieved (Figs 2 & 3), and experiments with cells in culture showed that this can be accomplished with no apparent toxic effect to surrounding cells (Fig 4). More importantly, the levels of drug that can be achieved in cell culture systems are enough to be prophylactic, preventing primary infection with different strains of HSV-1 and HSV-2 (Fig 5).

Previous studies have displayed evidence that both silicone and EVA exhibit characteristics needed to act as vehicles for controlled release devices when combined with drugs in a matrix-based device [20, 21]. These polymers, which have both been used in FDA-approved devices [12, 13], are non-biodegradable, making them good candidates for insertion into the vagina for an extended, yet finite, amount of time. Also, the polymers are relatively elastic compared to other polymers, allowing for patient comfort and hopefully lowering the possibility of poor patient compliance.

Ideally these types of vaginal rings should distribute consistent amounts of drug over the course of one month. Since we modeled our rings after contraceptive rings that consistently deliver drug over that time period, we hoped to achieve similar release kinetics from our rings to reduce issues with replacement and patient compliance [9, 12, 13].

We expect that next steps will include in vivo experimentation using a small animal model. We will then be able to compare whether our rings are safe and effective in a more relevant milieu than simple in vitro testing. Note that the rings designed in this study were made specifically for use in a small animal model; our current rings have about 12 times less surface area than that of a human NuvaRing [17]. We would expect that increasing the ring size to fit a human, at 65% ACV:35% EVA25, should increase drug release substantially.

Finally, we recognize that the use of EVA for intravaginal rings is not novel in and of itself [22, 23]; this polymer is currently successfully deployed as a drug delivery vehicle for antiretrovirals [22, 23] and contraceptives [15, 17]. Furthermore, polyurethane intravaginal rings are being explored for ACV delivery intravaginally [24]. Nonetheless, in contrast to these other implementations (typically drug reservoir systems), it is important to note that the method by which these rings were fabricated is relatively straightforward, requires inexpensive materials, and can be performed in virtually any basically equipped laboratory across the world. We hope that by creating these rings in a cost-effective manner, any end products could be produced or brought to any areas where transmission of HSV is a problem.

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References

- [1]. Whitley RJ, & Roizman B (2001). Herpes simplex virus infections review. *Lancet*. 10.1016/S0140-6736(00)04638-9
- [2]. de Miranda P, & Blum MR (2012). Pharmacokinetics of acyclovir after intravenous and oral administration. *Journal of Antimicrobial Chemotherapy*. 10.1093/jac/12.suppl_b.29
- [3]. Celum C, Wald A, Lingappa JR, Magaret AS, Wang RS, Mugo N, ... Corey L (2010). Acyclovir and Transmission of HIV-1 from Persons Infected with HIV-1 and HSV-2. *New England Journal of Medicine*. 10.1056/nejmoa0904849

- [4]. Laskin OL, Longstreth JA, Saral R, de Miranda P, Keeney R, & Lietman PS (1982). Pharmacokinetics and tolerance of acyclovir, a new anti-herpesvirus agent, in humans. *Antimicrobial Agents and Chemotherapy*. 10.1128/AAC.2L3.393
- [5]. Acosta EP, & Flexner C (2011). Antiviral Agents (Nonretroviral). In Goodman & Gilman 's The Pharmacological Basis of Therapeutics.
- [6]. Vere Hodge RA, & Field HJ (2013). Chapter One - Antiviral Agents for Herpes Simplex Virus. In *Antiviral Agents*.
- [7]. Semenkow SL, Johnson NM, Maggs DJ, & Margulies BJ (2014). Controlled release delivery of penciclovir via a silicone (MED-4750) polymer: kinetics of drug delivery and efficacy in preventing primary feline herpesvirus infection in culture. *Virology Journal*, 11(1), 34 10.1186/1743-422X-11-34 [PubMed: 24558980]
- [8]. Berkower CL, Johnson NM, Longdo SB, McGusty-Robinson SO, Semenkow SL, & Margulies BJ (2013). Silicone-Acyclovir Controlled Release Devices Suppress Primary Herpes Simplex Virus-2 and Varicella Zoster Virus Infections In Vitro. *Advances in Pharmacological Sciences*, 2013, 1–9. 10.1155/2013/915159
- [9]. Stegman JR, Badin JK, Biles KA, Etienne T, Fartash-Naini S, Gordon AD, ... Margulies BJ (2018). Volatile Acid-Solvent Evaporation (VASE): Molecularly Homogeneous Distribution of Acyclovir in a Bioerodable Polymer Matrix for Long-Term Treatment of Herpes Simplex Virus-1 Infections. *Journal of Drug Delivery*. 10.1155/2018/6161230
- [10]. Owen DH, & Katz DF (1999). A vaginal fluid simulant. *Contraception*. 10.1016/S0010-7824(99)00010-4
- [11]. Kalu NN, Desai PJ, Shirley CM, Gibson W, Dennis PA, & Ambinder RF (2014). Nelfinavir Inhibits Maturation and Export of Herpes Simplex Virus 1. *Journal of Virology*. 10.1128/jvi.03790-13
- [12]. Gonzalez-Calle A, Brant R, Diniz B, Swenson S, Markland F, Humayun MS, & Weiland JD (2018). Disintegrin-Integrin Binding for Attachment of Polymer Substrate to the Retina. *Journal of Clinical & Experimental Ophthalmology*. 10.4172/2155-9570.1000752
- [13]. Borhani S, Hassanajili S, Ahmadi Tafti SH, & Rabbani S (2018). Cardiovascular stents: overview, evolution, and next generation. *Progress in Biomaterials*. 10.1007/s40204-018-0097-y
- [14]. Biron KK (2007). Candidate anti-herpesviral drugs; mechanisms of action and resistance. In *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*. 10.1017/CBO9780511545313.069
- [15]. Brache V, & Faundes A (2010). Contraceptive vaginal rings: A review. *Contraception*. <https://doi.org/10.1016/j.contraception.2010.04.012>
- [16]. Mulders TMT, & Dieben TOM (2001). Use of the novel combined contraceptive vaginal ring NuvaRing for ovulation inhibition. *Fertility and Sterility*. 10.1016/S0015-0282(01)01689-2
- [17]. National Center for Biotechnology Information PubChem Database. (n.d.). NuvaRing, CID=9960701 Retrieved May 30, 2019, from <https://pubchem.ncbi.nlm.nih.gov/compound/Nuvaring>
- [18]. Keeney RE, Kirk LE, & Bridgen D (1982). Acyclovir tolerance in humans. *The American Journal of Medicine*. 10.1016/0002-9343(82)90086-9
- [19]. National Institutes of Health. (2009). Why Genital Herpes Boosts the Risk of HIV Infection. Retrieved May 30, 2019, from <https://www.nih.gov/news-events/nih-research-matters/why-genital-herpes-boosts-risk-hiv-infection>
- [20]. Beutner KR (1995). Valacyclovir: a review of its antiviral activity, pharmacokinetic properties, and clinical efficacy. *Antiviral Research*. 10.1016/0166-3542(95)00066-6
- [21]. Ramadevi A, Padmavathy T, Stigall G, Paquette D, & Kalachandra S (2008). EVA copolymer matrix for intra-oral delivery of antimicrobial and antiviral agents. *Journal of Materials Science: Materials in Medicine*. 10.1007/s10856-007-3109-3
- [22]. Singer R, Mawson P, Derby N, Rodriguez A, Kizima L, Menon R, ... Zydowsky TM (2012). An intravaginal ring that releases the NNRTI MIV-150 reduces SHIV transmission in macaques. *Science Translational Medicine*. 10.1126/scitranslmed.3003936

- [23]. Mesquita PMM, Rastogi R, Segarra TJ, Teller RS, Merna Torres N, Huber AM, ... Herold BC (2012). Intravaginal ring delivery of tenofovir disoproxil fumarate for prevention of HIV and herpes simplex virus infection. *Journal of Antimicrobial Chemotherapy*. 10.1093/jac/dks097
- [24]. Moss JA, Malone AM, Smith TJ, Kennedy S, Kopin E, Nguyen C, ... Baum MM (2012). Simultaneous delivery of tenofovir and acyclovir via an intravaginal ring. *Antimicrobial Agents and Chemotherapy*. 10.1128/AAC.05662-11

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Figure 1. Size comparison of 65% ACV (w:w) EVA25 ring to the size of an American penny.
The photo was taken using an iPhone 6S; final image was processed with Photoshop.

Figure 2. Seven day release of ACV from devices.

Silicone (MED-4750), EVA25, and EVA40 were tested for drug release at 50% (w:w) drug load. Each device was placed in 1 mL of VFS at 37 °C. Each day for seven consecutive days, the VFS was removed and saved for HPLC analysis, then replaced with 1 mL of VFS. Each experiment was conducted in triplicate. All values are relative to day zero.

Figure 3. Thirty days of ACV release from EVA devices.

EVA25 containing 65% ACV (w:w) or no drug, and EVA40 containing 65% ACV (w:w) or no drug, were analyzed for drug release kinetics over 30 days in VSF as in Fig 2. Each experiment was conducted in triplicate as in Fig 2. All results were quantified via HPLC and are relative to day zero.

Figure 4. In vitro cell viability.

An MTT assay was conducted to determine the safety of EVA25 rings in vitro. Viability was measured on mock-infected cells in α -MEM alone, with EVA25 rings containing no drug, with 65% ACV (w:w) EVA25 rings, or with 50 μ g/mL ACV solution added to media. Each experiment was performed four times with three replicates each ($p=0.9$).

Figure 5. In vitro suppression of primary HSV infection.

In vitro infection assays were performed to analyze the suppressive capabilities of the experimental rings compared to normally infected Vero cells with no treatment. Vero cells were either mock-infected (data not shown) or infected with either **(A)** HSV-1 (17); **(B)** HSV-1 (KOS); **(C)** HSV-2 (MS); or **(D)** HSV-2 (G) in the presence of no treatment, EVA25 rings, 65% ACV (w:w) EVA25 rings, or 50 µg/mL ACV diluted in α -MEM. Titers are expressed as log (pfu/mL) for each infection/treatment combination. Experiments were performed in triplicate.