Zinc oxide tetrapods inhibit herpes simplex virus infection of cultured corneas

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Purpose: Infection of the human cornea by herpes simplex virus type-1 (HSV-1) can cause significant vision loss. The purpose of this study was to develop an ex vivo model to visualize viral growth and spread in the cornea. The model was also used to analyze cytokine production and study the antiviral effects of zinc oxide tetrapods.

Methods: A β-galactosidase-expressing recombinant virus, HSV-1(KOS)tk12, was used to demonstrate the ability of the virus to enter and develop blue plaques on human corneal epithelial (HCE) cells and corneal tissues. Freshly obtained porcine corneas were cultured and then scratched before infection with HSV-1(KOS)tk12. The blue plaques on the corneas were imaged using a stereomicroscope. Western blot analysis for HSV-1 proteins was performed to verify HSV-1 infection of the cornea. Using the ex vivo model, zinc oxide tetrapods were tested for their anti-HSV-1 potential, and a cytokine profile was developed to assess the effects of the treatment.

Results: Cultured corneas and the use of β-galactosidase-expressing HSV-1(KOS)tk12 virus can provide an attractive ex vivo model to visualize and study HSV-1 entry and spread of the infection in tissues. We found that unlike cultured HCE cells, which demonstrated nearly 100% infectivity, HSV-1 infection of the cultured cornea was more restrictive and took longer to develop. We also found that the zinc oxide tetrapod–shaped nano- and microstructures inhibited HSV infection of the cultured cells, as well as the cultured corneas. The cytokine profile of the infected samples was consistent with previous studies of HSV-1 corneal infection.

Conclusions: The ability to visualize HSV-1 growth and spread in corneal tissues can provide new details about HSV-1 infection of the cornea and the efficacy of new cornea-specific antiviral drug candidates. The ex vivo model also demonstrates antiviral effects of zinc oxide tetrapods and adequately portrays the drug delivery issues that cornea-specific treatments face.

Herpes simplex virus 1 (HSV-1), a member of the Alphaherpesvirinae subfamily, contains a double-stranded linear DNA genome, which is encapsulated by a protein capsid and a host-derived envelop [1]. The other serotype, HSV-2, is closely related, but it has been shown that HSV-1 is responsible for more than 95% of cases of ocular herpes. Most notably, HSV-1 is the primary cause of infectious blindness in developed nations due to the virus’s ability to remain latent in neuronal cell bodies. The latent state of HSV-1 is characterized by the loss of viral protein synthesis via entry of a state of non-viral genome replication. The HSV-1 infectious or lytic state can be reactivated due to the presence of environmental cues, such as increased host stress levels or loss of immunity [2]. In the United States alone, it is estimated that 20,000 new cases of ocular HSV-1 infection occur annually with an additional 28,000 cases of reactivation reported during the same period [3]. Thus, generating new models that can add more details about the HSV-1 infection mechanism in the cornea can be vital for developing new understanding of the viral pathogenesis and more effective drug treatments.

A promising area of antiviral drug development lies in exploiting HSV-1 entry into cells via various cellular membrane receptors. HSV-1 contains multiple glycoproteins on its envelope that facilitate entry. Some of these glycoproteins, namely, gB, gD, gH, and gL, are necessary for infection to occur [4]. HSV initiates entry via interactions of gB and gC to heparan sulfate proteoglycans expressed on corneal epithelial cell membranes. This results in the virus attaching to cells. Once attached, the viral gD binds to its cognate host receptor, nectin-1 or 3-O sulfated heparan sulfate, which is required for genome-containing capsid penetration into epithelial cells. Furthermore, the genome-containing capsid then travels via the cellular microtubule network to the nucleus for replication in the lytic cycle or insertion into the normal cell genome in the lysogenic cycle [5-7]. Given the presence of various pockets of positively charged amino acids on these viral glycoproteins, developing antivirals that
contain negatively charged molecules has yielded promising results [8]. One such antiviral material candidate is zinc oxide [9]. The uniquely designed zinc oxide tetrapod structures contain engineered oxygen vacancies and therefore, are highly negatively charged. They block infection of cultured cells by HSV-1 [9] and by HSV-2 [10,11]. However, the zinc oxide tetrapods have not been tested for their ability to block HSV-1 infection of the corneal cells and the cornea.

One promising way to study viral infection in the cornea is based on ex vivo cultures of the cornea. Cultured corneas can provide a useful model for studying HSV infection [12]. They can mimic many key aspects of the in vivo tissue environment and more accurately depict viral spread than cultured cells. To add novelty to the cultured cornea infection model, our study uses a recombinant HSV-1 KOS viral strain that incorporates the Escherichia coli lacZ gene (Gene ID: 945006, OMIM: 230500) [13] as a reporter system for viral infection. A product of lacZ, β-galactosidase, can easily be measured qualitatively and quantitatively by the addition of substrates, such as 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) or O-nitrophenyl β-D-galactopyranoside (ONPG), to the tissue culture media. This allows for easy visualization of HSV-1 infection [14]. Our study shows that the growth of infection in the corneal tissues can be visualized, and the model can be used to demonstrate the antiviral efficacy of zinc oxide tetrapods.

**METHODS**

**Cells and virus:** Human corneal epithelium (HCE) cell line was provided by Dr. Kozaburo Hayashi, National Eye Institute, Bethesda, MD [15, 16]. HCE cell line was grown in Minimal Essential Media (MEM, Life Technologies, Carlsbad, CA) and augmented with 10% fetal bovine serum (FBS, Sigma Aldrich, St Louis, MO) and 1% penicillin and streptomycin (P/S, Life Technologies). African green monkey kidney (VERO) cell line was supplied by P.G. Spear, Northwestern University, Chicago, IL. This cell line was grown with Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) augmented with 10% FBS and 1% P/S. VERO cells were utilized to propagate and titrate KOS-tk12 virus.

To assess HSV-1’s ability to utilize β-galactosidase reporter assay the HSV-1 strain KOS-tk12 was used. This is a recombinant virus with bacterial lacZ gene (Gene ID: 945006, OMIM: 230500) inserted into HSV-1’s thymidine kinase gene (Gene ID: 2703374, OMIM: 230500). The product of lacZ is β-galactosidase. This gene is under control of HSV-1 infected cell protein 4 (ICP4) promoter [13]; 106 plaque forming units (PFU) were used to infect the pig corneal epithelia, and 0.1 multiplicity of infection (MOI) was used for infecting HCE cells.

**Short tandem repeat (STR) analysis:** To confirm the authenticity of the cell lines used in this study, STR analysis was performed at the DNA Services Facility at the University of Illinois at Chicago. The cell lines were confirmed by using GenePrint 10 system Kit (Promega, Madison, WI). The kit amplifies nine STR loci plus the gender determining locus, Amelogenin. After the amplification samples were analyzed using ABI Prism® 3730x1 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the data was analyzed using GeneMapper® v5.0 software (Applied Biosystems). Appropriate positive and negative controls were used throughout the test procedure.

**Corneal epithelium processing:** Porcine eyes were donated from Park Packing Co, Chicago, IL on day of sacrifice. Protocol was adapted from [17] as follows to achieve best results. To optimize ex vivo infection, corneas were excised same day as obtained. Tools to excise the corneas were sterilized in hot water, alcohol, and phosphate buffered saline (PBS 1X; 2.7 mM KCl, 1.5 mM KH2PO4, 138 mM NaCl, 8 mM Na2HPO4,7H2O, pH:7.2 Life Technologies). Corneas were excised, washed once with PBS, and then incubated overnight at 37 ºC in MEM media augmented with 5% antibiotic-antimycotic (Sigma Aldrich) and 1% insulin-transferrin-sodium selenite (Sigma Aldrich). Prior to infection, cornea were scarred via a 23G1 needle with three vertical and three horizontal lines in order to facilitate viral entry into epithelium. Corneas were excised in a variety of ways to determine maximum efficacy of infection as well as assayed in a variety of ways to confirm infection on a mRNA and protein level.

**Viral entry assay:** The entry assay was performed as outlined [18]. The ex vivo cornea epithelium and HCE cells were plated on six-well plates (one cornea per well, 1 × 10⁶ HCE cells per well) and then infected.

Entry was qualitatively confirmed in pig corneal epithelia and HCE cells via β-galactosidase assay interaction with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Life Technologies) resulting in blue color. Tissue and cells were infected with HSV-1 KOS-tk12 strain mixed in Opti-MEM (Life Technologies) and then incubated at 37 ºC. Two hours post infection (hpi), they were then washed with PBS and incubated in growth media (cornea media-corneas, MEM media-HCE cells) for 48-72 h (corneas) or 18-24 h (HCE cells) at 37 ºC. Fixation, permeabilization and staining were adapted from [10] as follows. Tissue and cells were incubated in fixing solution (2% formaldehyde, 2% glutaraldehyde) overnight (corneas) or 20 min (HCE cells). Tissues and cells were incubated in permeabilization solution
(2 mM MgCl₂, 0.01% deoxycholate, 0.02% NP-40) overnight (corneas) or 20 min (HCE cells). Tissues and cells were incubated in staining solution (PBS, K3Fe(CN)₆, K4Fe(CN)₆, 1mg/ml X-gal substrate) overnight to twenty-four hours (corneas) or one hour to overnight (HCE cells). Between each step, tissue and cells were washed with PBS. Visualization of blue color was confirmed by imaging the corneal tissue under the SteREO Discovery V20 microscope (Carl Zeiss, Germany, Jena, Germany). For HCE cells, the blue color was visualized under Axiovert 200 microscope (Carl Zeiss). The amount and intensity of the blue color were quantified via Metamorph Microscopy Automation and Image Analysis Software (Sunnyvale, CA).

Zinc oxide development and viability as antiviral on infected ex vivo pig corneal epithelium: Zinc oxide tetrapod–shaped nano- and micro-structures were developed by the flame transport synthesis method [19]. This method requires mixing of zinc metal micropowder with sacrificial polymer powder and burning mixture in a muffle box furnace at 900 °C for around 30 min. This resulted in tetrapod structures with arm diameters from 200 nm to 1 μm and arm lengths from 5 μm to 30 μm, as confirmed with scanning electron microscopy (SEM). Additional details about the growth of tetrapod-shaped structures from zinc oxide have already been described in previous work. The zinc oxide tetrapods (ZnOTs) used here were consistent throughout the experimentation as an antiviral to HSV-1. All zinc oxide tetrapods used in the experiments were not treated with ultraviolet (NUV) [11].

NUV ZnOTs were used to assess antiviral ability against the HSV-1(KOS)tk12 virus on the ex vivo pig corneal epithelium (and the in vitro HCE cells). The neutralization protocol was as follows. ZnOTs at a concentration of 1 mg/ml (0.1 mg/ml, HCE cells) were mixed in solution with the HSV-1(KOS)tk12 virus at a titer of 10⁵ (0.1 MOI, HCE cells) and Opti-MEM. The solution was then mixed via brief vortex spins and pipetting and incubated at room temperature for 20 min. One milliliter of the neutralization solution was pipetted on HCE cells. The infection solution remained on the corneas or HCE cells for approximately 2 h, and then the corneas were washed with 1X PBS and incubated with 2 ml of corneal solution (MEM solution, HCE cells) for 48–72 h post infection (18–24 hpi, HCE cells). Western blot, quantitative PCR (qPCR), and β-galactosidase assay were used to assess the efficacy of the neutralization treatment on the infection ability of the HSV-1(KOS)tk12 strain.

Quantitative polymerase chain reaction: To detect real-time viral transcript levels, qPCR was performed. Briefly, pig corneas were washed with PBS before the epithelial cells were gently scraped off into TRIzol (Life Technologies) using a sterile surgical scalpel. For the HCE cells, the cells were washed with PBS before TRIzol was added. RNA extraction was followed according to the TRIzol protocol, and cDNA was made using the protocol and conditions from the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Using primers for the cytokines interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α), interferon-alpha (IFN-α), and interferon-beta (IFN-β), a qPCR was performed as per the Fast SYBR Green protocol (Applied Biosystems). The qPCR conditions were as follows: Enzyme Activation: 95 °C, 20 s (cycles - hold), Denature: 95 °C, 1 s (40 cycles) and Anneal: 60 °C, 20 s (40 cycles).

Immunoblotting: To assess the viral infectivity of the HSV-1 strain on HCE cells and corneal epithelial tissue, samples were prepared for western blot analysis. At the indicated times post infection, epithelial cells from pig corneas were gently scraped into radioimmunoprecipitation buffer (RIPA, Sigma Aldrich) containing a protease-phosphatase inhibitor cocktail (Halt, Thermo Fisher, Waltham, MA) using a sterile surgical scalpel, incubated on ice for 30 min, and centrifuged at 16,000 ×g for 15 min at 4 °C to pellet out any cell debris. The supernatants were denatured and electrophoresed on a 4–12% Bis-Tris NuPAGE Gel (Thermo Fisher). Using a dry blotting system (iBlot, Life Technologies), proteins from the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 60 min in 5% non-fat milk in Tris buffered saline containing 0.1% Tween-20 (TBS-T) followed by overnight incubation with primary antibodies against HSV-1 gD and gB (Abcam, Cambridge, MA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C. The following day, the membranes were washed with TBS-T before they were incubated with horseradish peroxidase conjugated secondary antibodies for 60 min at room temperature. The membrane was developed by the addition of Femto-Sensitivity ECL substrate (Thermo Fisher). Chemiluminescence was detected and imaged on ImageQuant LAS4000 (GE Life Sciences, Pittsburg, PA). The HCE cells from the dishes were washed with PBS, scraped using cell scrapers (Thermo Fisher), pelleted by centrifugation, and resuspended in RIPA buffer containing protease-phosphatase inhibitor cocktail. Proteins from these samples were detected as mentioned above.

Statistical analysis: All corneal culture experiments were performed in triplicates to confirm consistency of results. The Student T-test statistical analysis was conducted via the use of GraphPad Prism software (La Jolla, CA). A p value of
RESULTS

HSV-1(KOS)tk12 infection of human corneal epithelial cells: To test the effectiveness of the HSV-1(KOS)tk12 strain (pfu 10⁶) to infect cells of the corneal epithelia, human corneal epithelial cells were used [20]. Viral entry was verified via the presence of blue coloring in the cells due to activation of the X-gal substrate by β-galactosidase, 24 hpi. Confirmation of infection via β-galactosidase has the benefit of staining only cells that are actively producing the reporter enzyme expressed via the incoming viral genome (i.e., β-galactosidase) or in other words, productively infected by HSV-1. This decreases false positives seen with other reporter virus assays, such as those that use fluorescent viruses that may not well distinguish between membranes bound and entered viruses. As shown in Figure 1A (blue cells, right panel), nearly 100% of the HCE cells were infected by the recombinant virus.

Next, to confirm the productive infection of the HCE cells by the recombinant virus, we probed for HSV glycoproteins gB and gD, which are HSV late gene products. As shown in Figure 1B, gB and gD were not present in the uninfected HCE wells, whereas statistically significant quantities of gB and gD were seen in the wells infected with the virus, indicating that the recombinant virus causes a productive infection in HCE cells. Quantification of the protein was performed via western blot analysis, and the results were normalized against corresponding GAPDH bands (Figure 1C). These results demonstrate that more gD than gB was produced by the infected HCE cells. Taken together, the results from the β-galactosidase assay and the presence of quantifiable amounts of viral glycoproteins, gB, gD confirm that HSV-1(KOS)tk12 is effective in entering and proliferating in HCE cells.

HSV-1(KOS)tk12 infection of ex vivo corneal epithelium: To determine the efficacy of HSV-1(KOS)tk12 in tissue cultures, ex vivo corneal epithelial tissue, extracted pig corneas were used [12,17,21,22]. As outlined in Figure 2A, the most novel part of our approach was the use of X-Gal recombinant virus to infect the cultured corneas and ensure that blue plaques can be seen on the infected corneas. In multiple experiments, we consistently observed infection by the recombinant virus and the spread of infection in a dendritic fashion, which is considered a hallmark of HSV infection of the cornea. As shown in Figure 2B, the central portion of the infected section (boxed) did not have many blue cells. Looking at the morphology of that area, it was clear that the loss of the blue cells originated from dead cells or scarred tissue in that area. To confirm the infection, the presence of the viral glycoprotein gB was examined and quantified via western blot analysis of the cornea cell lysates, which verified the infection and presence of HSV proteins in the infected corneas (Figure 2C).

Zinc oxide tetrapods block HSV infection: NUV-treated zinc oxide tetrapods were produced using the flame synthesis method. This method creates tetrapods with a wide range of applicability, allowing them to be used with high accuracy and no substrate limitation [19]. NUV zinc oxide tetrapods were used to test antiviral properties against the HSV-1(KOS)tk12 strain virus. Neutralization treatment, in which virus and the ZnOTs were incubated before infection, statistically significantly reduced the amount of β-galactosidase expression, as evidenced by the decreased presence of blue staining in the pig cornea epithelium and the HCE cells. Samples infected with the zinc oxide/HSV-1(KOS)tk12 virus showed statistically significantly less blue coloring in the in vitro and ex vivo samples compared with the samples infected with HSV-1(KOS)tk12 alone (Figure 3B, Figure 4A). Quantification of X-gal via the amount of blue present in these samples was completed by using MetaMorph software to compare the infection visualized infected versus the infected plus ZnOT-treated samples. This represented a more objective method for determining the ability of zinc oxide to combat HSV-1 insult than simple visual assessment (Figure 3C, Figure 4B). To assess the antiviral effect of the NUV zinc oxide tetrapods on proteins necessary for the spread of HSV-1, quantification of viral glycoproteins, gB (HCE cells) and gD (corneas), was undertaken. The infected samples were compared with the infected plus zinc oxide tetrapod–treated samples to assess the levels relative to GAPDH. Consistent with the decrease seen in β-galactosidase expression, these glycoproteins were statistically significantly reduced in the in vitro (HCE cells) and ex vivo (cornea tissue) neutralization treatment samples (Figure 3D, Figure 4C).

Cytokine profile of infected ex vivo corneal epithelial tissue: To assess the immune response initiated by the corneal epithelial tissue, the mRNA levels of cytokines known to play a role in this response was analyzed via qPCR analysis. In particular, the levels of the cytokines IL-6, IL-8, TNF-α, IFN-α, and IFN-β were examined for uninfected versus infected and zinc oxide versus zinc oxide plus infected samples to compare the baseline level of the cytokines in the control samples with that in the infected samples. Results showed a statistically significant change in the cytokine levels in the infected samples (Figure 5). However, not all results were consistent with the expected outcome based on previous literature analysis, namely, that of IL-6 [23].

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Figure 1. Infection of HCE cells with the HSV-1(KOS)tk12 virus. A: Viral entry assay using the 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) substrate to confirm entry. Viral entry is confirmed by the prevalent blue observed in the infected human corneal epithelial (HCE) cells (blue, right panel). B: A representative immunoblot of the cell lysates for HSV-1 glycoproteins, gB and gD in uninfected and infected HCE cells. C: Quantification of gD and gB viral protein levels of representative immunoblot.
Figure 2. Development of ex vivo pig corneal model for HSV-1(KOS)tk12 infection. A: General schematic of major steps undertaken to develop pig cornea cultures harvested from pigs aged 180-200 days. B: Representative images taken via a modular stereo microscope of uninfected and infected cornea treated with the 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) substrate. C: Representative HSV-1 gB immunoblot (left panel) and intensity quantification (right panel) present in uninfected and infected corneal epithelial cell lysates.
Figure 3. Analysis of the antiviral, zinc oxide tetrapods in mitigating HSV-1(KOS)tk12 infection in HCE cells. A: Scanning electron microscope image of zinc oxide tetrapod nanoparticles. B: Representative bright-field microscopic images of infected and zinc oxide-treated infected HCE cells to assess viral entry via the 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) substrate. C: Quantification of viral entry and spread assessed by amount of blue present via Metamorph software. Significance was determined by t-test: p<0.0001. D: Representative immunoblot image (left panel) and its quantification (right panel) for HSV-1 gB from cell lysates of uninfected and zinc oxide-treated infected HCE cells. Note: data used for the immunoblot analysis and quantification of cell lysates in panel D were taken from HCE cells infected with the wild strain herpes simplex virus type-1 (HSV-1)KOS virus.
Figure 4. Analysis of zinc oxide tetrapods in mitigating HSV-1(KOS)tk12 infections in pig corneas. A: Representative modular stereo microscope imaging of infected and zinc oxide-treated infected pig corneas to assess viral entry and spread via 5-bromo-4-chloro-3-indoly1-β-D-galactoside (X-gal) substrate. B: Quantification of viral entry and spread assessed by amount of blue present via Metamorph software. Significance was determined by t-test: p<0.001. C: Representative immunoblot image (left panel) and quantification (right panel) of HSV-1 gD from untreated and zinc oxide-treated infected pig corneal epithelial cell lysates.
Figure 5. Analysis of innate immune response evidenced in pig corneas as depicted by cytokine and interferon profile of known innate response mediators. Comparison in cytokine profile of uninfected, zinc oxide-treated uninfected, infected and zinc oxide-treated infected cornea samples was undertaken to control for major variables tested, namely, antiviral, zinc oxide tetrapods treated and virus, herpes simplex virus type-1 (KOS)t12. Significance was determined by t-test: p<0.01.
DISCUSSION

Characterizing herpes simplex virus and its ability to infect corneal tissue is an important concern, as HSV-1 is known to cause keratitis, which results in loss of vision. Although usually treatable, corneal epithelial keratitis can become devastating causing dendritic ulceration, corneal neovascularization, scarring, and eventual blindness. This is due to the ability of HSV-1 to cause recurrent infection, a benefit of the virus’s life cycle in which primary infection can occur at the corneal epithelium during the lytic phase and secondary infection can occur at the trigeminal ganglia of the host during the latent viral phase. Catalysts can then cause reactivation of HSV-1 lytic infection at latently infected cell host sites [3,24,25]. Thus, there is a need to develop a model that not only facilitates testing of novel drugs in a timely manner but also accurately replicates the human host environment to study both viral cycles.

The purpose of this study was to further develop an ex vivo model that can be used to visualize the spread of HSV-1 infection. The ability to use the β-galactosidase reporter virus represents a novel step in ex vivo corneal tissue studies. β-galactosidase interaction with the X-gal substrate represents a highly specific and rapid method for confirming the active infection of corneal tissue with HSV-1 by visual inspection [14,26]. Additionally, β-galactosidase is not expressed during the latent viral phase but is reexpressed during viral reactivation in secondary infected cells, providing an avenue for studying the latency establishment and reactivation mechanisms of HSV-1 [27-29]. This is an important development in studying the ability of HSV-1 to penetrate corneal tissue, as use of reliable ex vivo tissue models is often more efficient and ethical in comparison to in vivo models. Given this, ex vivo models have been shown to be promising for developing next-generation methods of drug delivery to combat lytic and latent HSV-1 infection [30].

Assessment of HSV-1(KOS)tk12 to infect corneal epithelial cells was first undertaken in cell cultures in vitro. Infection was assessed by the blue present in infected cells, noting that HCE cells express the three major entry parts, namely the glycoproteins gB and gD. Both assays were consistent with active viral infection in HCE cells, as more blue and increased gB and gD glycoprotein levels were observed in the infected lanes. These results are consistent with previous studies that have defined the cornea as the gateway to HSV-1 infection of other eye parts, noting that HCE cells express the three major entry receptors (nectin-1, herpesvirus entry mediator, and paired immunoglobulin-like type 2 receptor α) needed to maximize viral entry [20,31].

After verifying the ability of HSV-1(KOS)tk12 to infect corneal cells in vitro, developing a competent model using this virus in ex vivo corneal tissue samples was undertaken. This undertaking required investigating the optimal combination of such variables as the methodology for excising the cornea from the eye, the viral incubation period, the scarring methodology, and the X-gal concentration used. It was determined that by keeping a scleral border around cornea epithelial tissue, a more robust infection occurred. This could be because not cutting into the cornea preserves the epithelial tissue to a higher degree, keeping cells alive during the course of infection. The incubation period for the excised corneas in the cornea media was varied to determine the time needed to adjust to the new environment. It was determined that overnight incubation led to the highest rate of successful infections. When the corneal epithelium was scarred to allow the virus to enter, light pressure applied to the needle with a still visible scarring pattern resulted in the optimal infection. This may be due to maintenance of the integrity of the epithelial cells below the first layer. HSV-1 has been shown to infect deeper layers of the corneal epithelium [3]. To assess the viral incubation time, multiple time points were employed, at 12 h intervals from 24 hpi to 72 hpi. Robust infection was seen between 48 and 72 hpi, as this allowed more time for HSV-1 to spread from the scarred epithelium. Finally, assessment of the X-gal concentration needed to optimize interaction with the β-galactosidase enzyme produced by HSV-1 infection of the corneal cells was assessed. Following the X-gal staining protocol used for cell cultures, a concentration of 1 mg/ml was sufficient to visualize blue staining in the ex vivo corneal tissue samples [28].

Next, the model was used to test the efficacy of a promising antiviral candidate, zinc oxide tetrapods. The tetrapods have been shown to block HSV-2 entry into human vaginal epithelial cells [11]. The negatively charged tetrapods compete for the binding of HSV to its negatively charged attachment receptor, heparan sulfate [32]. To determine whether zinc oxide tetrapods would exhibit similar antiviral properties against the HSV-1(KOS)tk12 strain, infectivity was first tested in human corneal epithelial cells. It was determined with the β-galactosidase reporter assay that zinc oxide effectively decreased viral infection when the virus was incubated with zinc oxide in a neutralization reaction (Figure 3B). Next, quantification of viral protein concentrations, namely, the glycoproteins gB and gD, which are necessary for viral entry was undertaken [18]. It was noted that these viral glycoproteins were statistically significantly reduced in the HCE cells treated with the neutralized HSV-1(KOS)tk12 virus. Thus, qualitative and quantitative assays have shown that zinc oxide tetrapods exhibit antiviral properties by
reducing the spread of infection and decreasing the production of the viral proteins necessary for secondary infection to occur. Finally, given that the in vitro corneal epithelial cells respond favorably to zinc oxide tetrapods, it was necessary to test the ability of the zinc oxide tetrapods against the ex vivo tissue model developed. The β-galactosidase reporter assay with X-gal and western blot analysis was undertaken to qualitatively and quantitatively determine the effect of zinc oxide tetrapods on viral infection and spread. Results were consistent with those seen in the HCE cells, with viral gB and gD levels statistically significantly lower in the pig corneas treated with the neutralized HSV-1(KOS)tk12 virus, as well as a notable reduction in the blue visualized in these samples (Figure 4).

To assess the innate immune response undertaken by ex vivo corneal epithelial tissue, qPCR was used to determine the cytokine profile at the mRNA level of the uninfected, infected, zinc oxide–treated, and neutralization-treated tissue samples. It was observed that the cytokines TNF-α and IL-8 and the type I interferons IFN-α and IFN-β were significantly increased in comparison with the uninfected ex vivo samples. It has been noted that transcriptional expression of IL-6, IL-8, and TNF-α is rapidly induced by activation of nuclear factor-kappa beta (NF-κB) in response to HSV-1 infection in in vitro cornea cell cultures [33]. These cytokines are known to facilitate acute immune response with chemotaxis of neutrophils and triggering of inflammatory and antigen-presenting cells in infected corneal tissue [23,34,35]. Furthermore, it has been noted that the corneal epithelial layer is the biggest producer of IL-6 in HSV-1-infected corneas [36]. However, this is in contrast with the qPCR data observed, in which the infected corneas showed a decrease in IL-6 levels and the neutralization-treated corneas corresponded to the higher IL-6 level. The neutralization result is particularly unique given the antiviral properties of the zinc oxide tetrapods, which would imply IL-6 levels closer to those of the uninfected samples. Given the ex vivo nature of the model and the timespan in which the mRNA levels were assessed (48 hpi), perhaps the lack of a robust IL-6 mRNA level is due to an insufficient incubation period to adequately characterize HSV-1 infection in the corneal epithelial tissue samples. Interestingly, HSV-1 has been shown to inhibit IL-6 production in HSV-1 infected leukocytes (macrophages) and HCE cells [37,38]. This occurs via various ways by silencing key players in the signal transduction pathways responsible for inducing IL-6 production [38-40]. In particular, micro-RNA miR-H6 inhibits ICP4 protein, an upregulator of early and late genes of active HSV-1 lytic infection in HCE cells. This has the side effect of also inhibiting IL-6 production [37].

Infection was also shown to increase the mRNA levels of the type I interferons (IFN-α and IFN-β). Activation of these interferons is noted as a primary defense mechanism to combat HSV-1 infection [41]. In vitro studies of HSV-1 infection on corneal epithelial cells have implied that this is due to a sequence of events initiated by toll like receptor (TLR) 3 activation due to the recognition of a specific viral signature pattern, which indirectly induces expression of IFN-β [42]. Furthermore, it has been noted that IFN-β can induce expression of IFN-α [43]. However, increased expression of IFN-α may not solely be explained by the induction in IFN-β. In vitro studies have noted that HSV-1 infection causes TLR7 induction, a known inducer of IFN-α [33]. The culmination of this evidence is important because it shows that ex vivo corneal epithelial tissue maintains a similar innate immune response pattern as seen in the in vitro models of HSV-1 infection.

A working ex vivo model of corneal epithelial tissue infection by the recombinant β-galactosidase expressing HSV-1 strain represents a useful tool for characterizing the virus and assessing the ability of new antivirals or drugs to combat HSV insult in corneal tissue [30]. The strength of this ex vivo model lies in its ability to mimic the in vivo tissue environment, and the fact that human and pig corneas demonstrate a high degree of similarity in tissue organization and cellular composition, including the highly homologous cells of the corneal epithelium [44]. Additionally, the inclusion of the β-galactosidase reporter assay using the (KOS) tk12 strain in cornea cultures is novel because of its ability to quickly and accurately evaluate the spread of infection. Most notably, this model represents a unique way to understand lytic infection of HSV-1, which can lead to viral latency in the sensory and autonomic ganglia, which in turn is responsible for the most devastating effects of the infection [31,45]. Future studies on HSV-1 insult using our model will be highly relevant on a global scale as HSV-1 infects 60% to 90% of the world’s population [46].

APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words “Appendix 1.”

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