World Journal of Advanced Research and Reviews

eISSN: 2581-9615 CODEN (USA): WJARAI

Cross Ref DOI: 10.30574/wjarr

Journal homepage: https://wjarr.com/

(Research Article)

Method for the growth of Herpes Simplex Virus (HSV-1 and HSV-2) in an oxygen-free environment

Balbina J. Plotkin *, Ira M. Sigar, Amber Kaminski, Alexis Borrelli, Victoria Zheng, Nicholas Schileru, Michelle Aelion and Brandon Siaj

Department of Microbiology and Immunology, College of Graduate Studies, Midwestern University, 555 31st St, Downers Grove, IL, USA; 60515.

World Journal of Advanced Research and Reviews, 2023, 18(01), 808–814

Publication history: Received on 03 March 2023; revised on 12 April 2023; accepted on 15 April 2023

Article DOI: https://doi.org/10.30574/wjarr.2023.18.1.0628

Abstract

Vaginal herpes infections occur under low oxygen conditions, including anoxia. Using an anaerobic (0% O_2) cell system, the infection rate, early protein expression, and progeny viability were determined. Anaerobic HeLa 229 cells (0% O_2; anaerobic DMEM; 24h) were infected with MOI 1 of HSV1/2, then incubated under aerobic (control) and anaerobic conditions. Infectivity (6h post-infection (pi)) was assessed by ONPG activity and enumerating X-gal-stained cells. HSV1/2 viral immediate-early entry, early entry, and late entry protein expression were determined by fluorescent antibody staining followed by HSV1/2 glycoprotein B mouse monoclonal antibody and DAPI. Statistical analysis was performed using t-test and data were considered significant when $p \leq 0.05$ (GraphPad Prism).

HSV1/2 infection under standard aerobic conditions was approximately twice that measured for anaerobically infected cells. In addition, the level of HSV-2 infectivity was significantly ($p < 0.05$) higher (~8-fold) than that of HSV-1, regardless of oxygen level. HSV entry stage protein expression (ICP8, ICP27, VP5) was detected for anaerobic HSV1/2. A similar pattern was observed for anaerobically grown progeny of HSV1/2, i.e., the number of progeny was higher when anaerobic progenies were grown under aerobic conditions (anaerobic to aerobic) as compared to those grown under anaerobic conditions (anaerobic to anaerobic). In addition, the progeny levels grown solely under anaerobic conditions trended higher for HSV-1 than HSV-2 progeny but were not significantly different. These findings show that HSV1/2 can infect and replicate in anaerobically growing cells and that oxygen is not essential for productive replication.

Keywords: Herpes; Anoxic; Anaerobic; Viral replication; Early protein expression

1. Introduction

Oxygen levels in the human body range from 0 to 13%, thus, infections arise in oxygen-limiting body regions where physioxia ranges from hypoxic to anoxic conditions [1, 2]. In addition, if inflammation occurs, such as when induced during active replication and lesion production by HSV1/2, there is a further reduction in oxygen levels [3, 4]. It is well-known that HSV geographically also infects body sites, e.g., the urogenital tract, that is minimally hypoxic to anoxic. Previous studies assessing cell growth under *in vitro* anoxic conditions (0-0.3% O_2) show that cell physiology, in the absence of oxygen as the terminal electron acceptor for the mitochondrial cytochrome oxidase system, differs from cell physiology when grown under CO_2-enriched atmospheric oxygen (21% O_2), i.e., a hyperbaric growth environment [5, 6, 7]. Since the subsequent decrease in oxygen tension, in addition to the pathogen's restriction to an intracellular environment, are indicators that HSV replicates in low oxygen concentrations *in vivo,* it is possible to gain insightful knowledge of the host-pathogen interactions involved under anaerobic growth conditions using the anaerobic cell model previously described to confirm that HSV1/2 anaerobic replication parallels that of aerobic growth [5, 6, 7]. The

* Corresponding author: Balbina J. Plotkin; Email: bplotk@midwestern.edu

Copyright © 2023 Author(s) retain the copyright of this article. This article is published under the terms of the Creative Commons Attribution Liscense 4.0.

focus of this study is to determine the methodology for growing HSV1/2 anaerobically and assess its replication characteristics compared to standard growth conditions (5% CO_2 in air).

2. Material and methods

2.1. HSV1/2 strains

Recombinant, entry proficient strains encoding a beta-galactosidase reporter of HSV-1 (KOS) gL86 and HSV-2 (KOS) 333gJ$^-$ activity were used for all assays [8, 9]. Virus stocks were maintained at -80°C until use, and virus from a single lot were used in this study.

2.2. Anaerobic HSV1/2 cell entry assay for both parental and progeny

HSV entry into HeLa cells was confirmed quantitatively by ONPG (*o*-nitrophenyl-β-D-galactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) assays post 6h incubation under anaerobic and aerobic conditions [10]. The level of virion infection over various concentrations was measured spectrophotometrically (20 min, OD_{405}) as previously described [10, 11]. The number of HSV-infected cells (MOI 1, X-gal staining of blue cells) on images (EVOS FL microscope) were counted manually using ImageJ. Statistical analysis was performed using a t-test two-sample. Data was considered significant when $p \leq 0.05$ (GraphPad Prism). All images were coded, and infected cells (blue-stained) manually counted by multiple observers under double-blinded conditions with inter-observer (n=4) reproducibility validated.

To infect the cells for both assays, HeLa 229 cells were seeded at 50% confluence of a 24 well plate (5% CO_2, 37 °C) at Day (-) 1 using Dulbecco's Modified Eagle Medium (DMEM; Corning), supplemented with glucose (4.5 g/L), L-glutamine (110 mg/L), 10% fetal bovine serum (FBS, VWR International), and 0.05 mg/L gentamicin (VWR International). At Day 0, plates were transferred to the anaerobic chamber (anaerobic gas mixture: H_2, N_2, CO_2; Whitley A35, Microbiology International), and media was changed to degassed antibiotic-free DMEM, confirmed to be anaerobic using dO_2 isopod oxygen electrode (eDAQ). Aerobic control plate media was changed to antibiotic-free DMEM, and incubated in air (5% CO_2, 37°C) [5]. On Day 1, the cells were infected [11].

2.3. HSV1/2 replication and progeny viability

To assess the viability of HSV1/2 progeny after anaerobic replication, HSV1/2 were inoculated onto replication permissive cell lines, i.e., African Green Monkey Kidney Epithelial (Vero) cells for HSV-2 and gL-expressing Vero (79B4) cells for HSV-1 [12, 13, 14, 15, 16]. Both cell lines were prepared as described above and on Day 0, Vero and 79B4 96 well plates were transferred to the anaerobic chamber. The medium was immediately changed to degassed antibiotic-free DMEM and confirmed to be anaerobic (dO_2 isopod oxygen electrode; eDAQ). Medium for the aerobic controls was changed to antibiotic-free DMEM, and incubated in air (5% CO_2, 37 °C). On Day 1, Vero and 79B4 cells were infected with HSV-1 and HSV-2, respectively, at MOI 1 and incubated for 24 h in homologous oxygen levels. On Day 2, the cell supernatants from Vero and 79B4 plates containing HSV-1 and HSV-2 progeny were removed by aspiration, pooled, and various dilutions were used to inoculate HeLa 229 cell plates (96 wells; anaerobic acclimated - Day 1 and aerobic control). The plates were then incubated for 6h under homologous, i.e., anaerobic to anaerobic and aerobic to aerobic (control) and heterologous anaerobic to aerobic and aerobic to anaerobic conditions. After 6 h post-infection, X-Gal staining was used to determine whether progeny from aerobic or anaerobically infected cells were viable, as previously described [11].

2.4. HSV1/2 viral entry protein fluorescent staining

HeLa 229 cells were seeded on coverslips at 80% confluence of a 24 well plate (5% CO_2, 37 °C) at Day (-) 1 using Dulbecco's Modified Eagle Medium (DMEM; Corning), supplemented with glucose (4.5 g/L), L-glutamine (110 mg/L), 10% fetal bovine serum (FBS, VWR International), and 0.05 mg/L gentamicin (VWR International). At Day 0, plates were transferred to Whitley A35 Anaerobic Chamber (anaerobic gas mixture: H_2, N_2, CO_2; Microbiology International), and media was changed to degassed low glucose antibiotic-free DMEM, confirmed to be anaerobic using dO_2 isopod oxygen electrode (eDAQ). Aerobic control plate media was changed to antibiotic-free DMEM, low glucose and incubated in air (5% CO_2, 37 °C). At Day 1, cells were infected with either HSV-1 or HSV-2 (MOI 1) and incubated for 7 h post-infection (pi). Every 15 minutes for 4 h pi, coverslips were washed 3x with PBS, and fixed with methanol. Primary antibodies used were HSV1/2 glycoprotein B mouse monoclonal antibody (SM3058P; Origene), HSV1/2 ICP27 Immediate-Early Entry monoclonal antibody (sc-69807; Santa Cruz Biotechnology), HSV-1 ICP8 Early Entry monoclonal antibody (sc-53329; Santa Cruz Biotechnology), HSV-2 ICP8 Early Entry monoclonal antibody (H2A024-100; Virusys Corporation), and HSV1/2 VP5 Late Entry monoclonal antibody (sc-13525; Santa Cruz Biotechnology). Secondary antibodies (Invitrogen)

used were Goat anti-Mouse IgG secondary antibody and Alexa Fluor 594 secondary antibody. Coverslips were mounted on slides with VECTASHIELD® Hardset™ Antifade Mounting Medium w/ DAPI (Vector Laboratories) [12-16]. Representative fluorescent images were obtained (Nikon Eclipse Ti2-E inverted fluorescence microscope).

2.5. Statistical analysis

Each infectivity experiment was conducted twice in octuplicate. Each imaging study was conducted twice in triplicate. Data were evaluated by analysis of variance with Tukey post hoc test, where appropriate (ANOVA; GraphPad InStat 3.10 for Windows, GraphPad Software Inc.) or unpaired t-test, where appropriate. Mean values were considered significantly different at $p \leq 0.05$.

3. Results and discussion

3.1. Effect of oxygen on HSV infective dose

Colonization of the vaginal mucosa by herpes viruses requires the ability to successfully infect and replicate within cells in an anaerobic environment [17]. While both HSV-1 and HSV-2 can cause vaginal infections with lesions, HSV-2 is most commonly associated with disease at this site [18, 19]. To date, the differences between HSV-1 and HSV-2 in their relative ability to cause vaginal infections has not been well characterized because until recently, no *in vitro* model system enabled the measurement of long-term anaerobic intracellular viral replication [5, 6, 7, 20]. Using a novel anaerobic (0% oxygen) cell growth model system developed by the Plotkin/Sigar Laboratory, the ability of HSV-1 and HSV-2 to anaerobically infect HeLa cells, express replication-associated early proteins, and produce viable progeny were determined. In this study, both HSV-1 and HSV-2 were capable of infecting HeLa 229 cells in the absence of oxygen (Fig 1). Initial levels of infectivity were screened by ONPG staining after growth under aerobic and anaerobic growth conditions. These findings indicated that there was no significant difference between aerobic and anaerobic infectivity for either HSV-1 or HSV-2 (Fig 1A). To verify these findings, quantification of the effects of oxygen on HSV infectivity was determined by direct observation of X-gal infected cells upon growth in the presence and absence of oxygen (Fig 1B). As was measured for ONPG staining, the level of HSV-2 cell entry was significantly higher than that measured for HSV-1 regardless of incubation condition, i.e., HSV-2 was 9.9-fold higher than HSV-1 under aerobic growth and 6.1-fold higher upon growth in the absence of oxygen. In addition, aerobic infection of HSV-2 was 3-fold higher than when grown anaerobically (Fig 1C), while HSV-1 aerobic infectivity was 1.8-fold higher under aerobic condition vs. anaerobic conditions. Although an MOI of 1 was used for viral dose and oxygen testing conditions regardless of HSV strain, the level of infection for HSV1/2 grown under aerobic condition was significantly higher than HSV-1 or HSV-2 grown anaerobically.

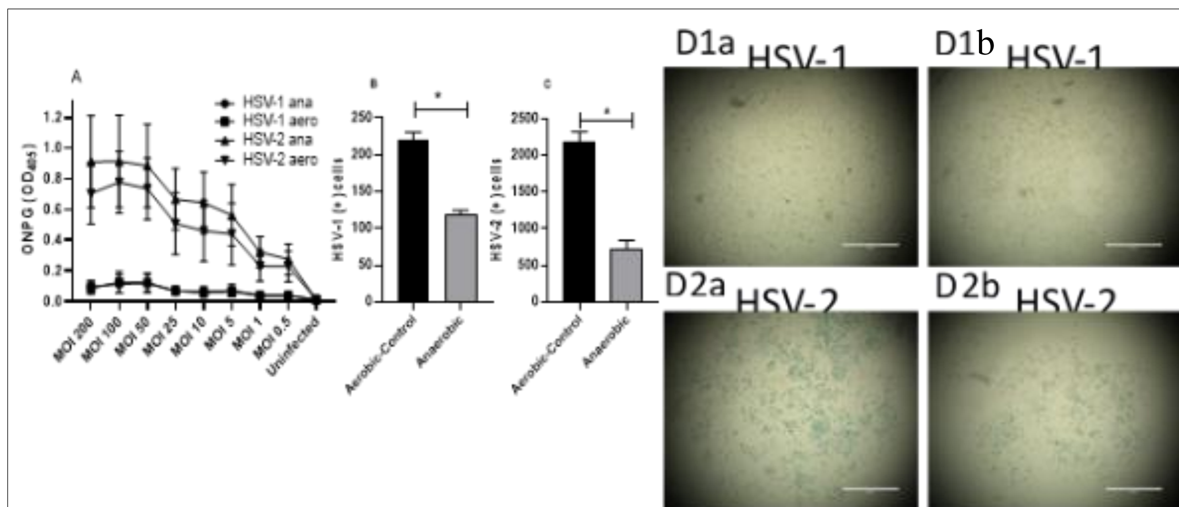
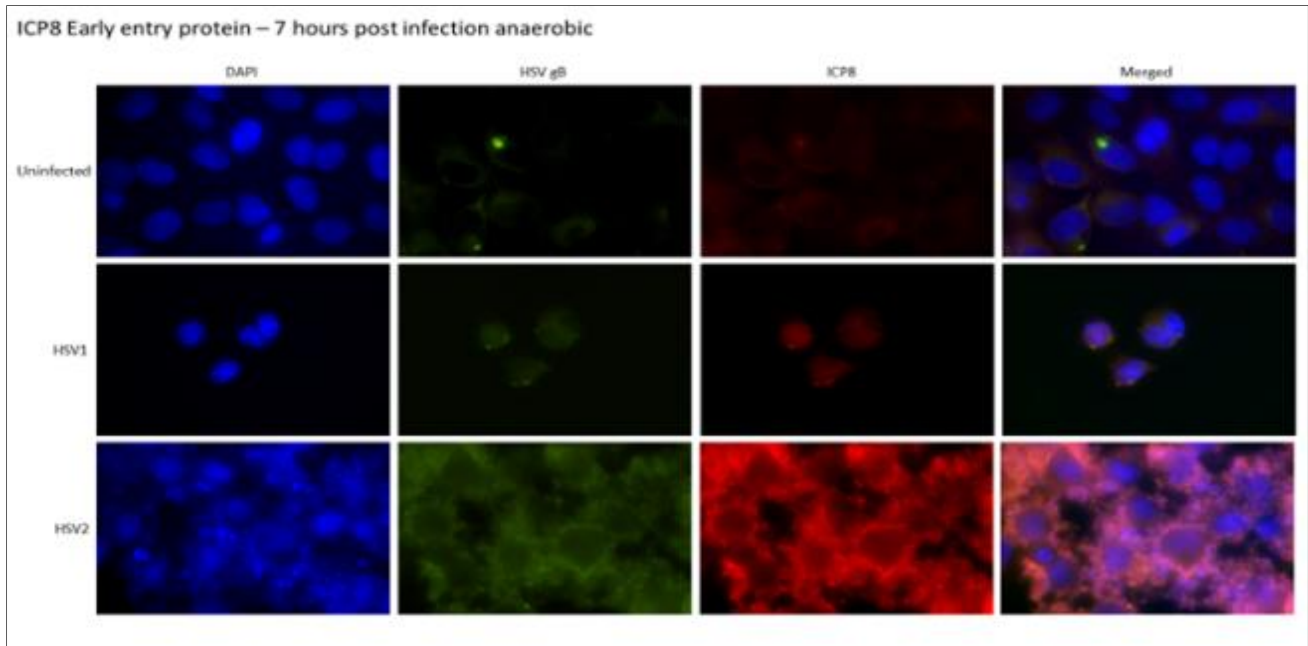
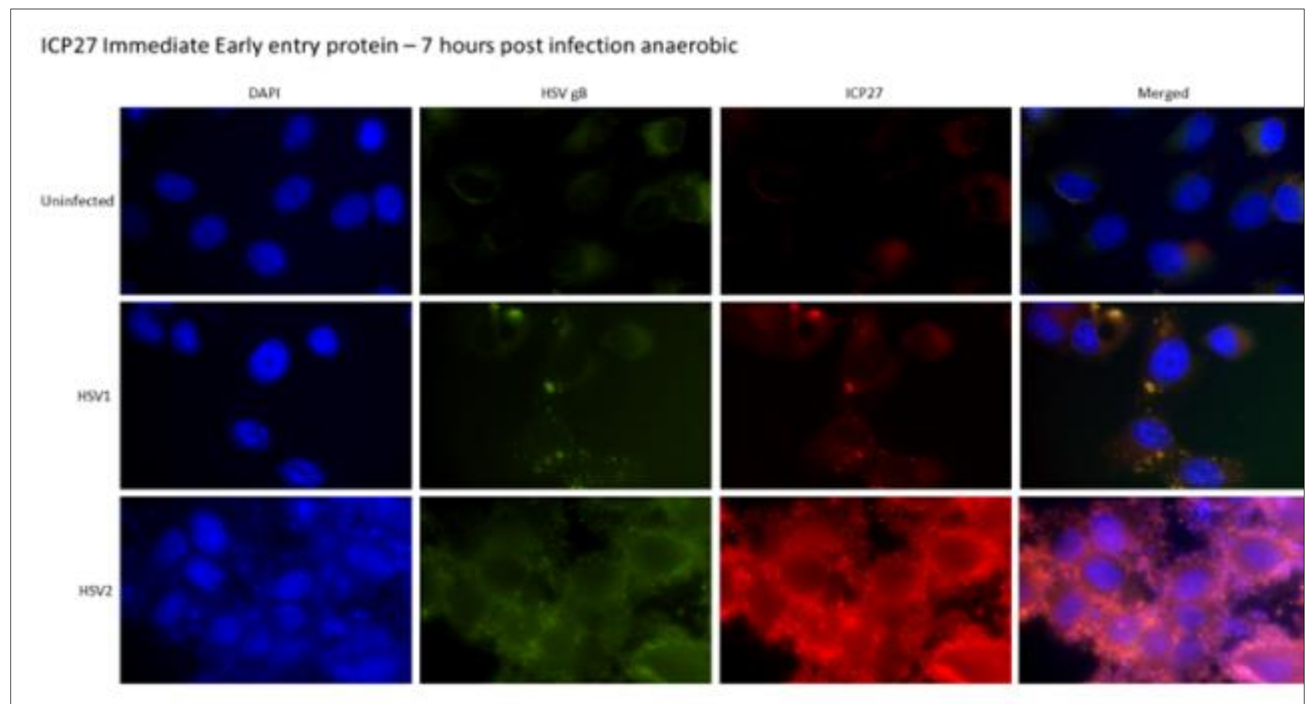


Figure 1 Aerobic and anaerobic HeLa cells infected with HSV-1 and HSV-2 post 6h incubation. (A) β-galactosidase levels in response to various MOI of HSV-1 and HSV-2 concentrations in the presence and absence of oxygen; (B,C) X-gal analysis of HSV-1/2 at MOI 1 positive cells in the presence and absence of oxygen; (D1a) Representative photomicrograph 10X of HSV-1 infected X gal-stained aerobic HeLa cells; (D1b) Representative photomicrograph 10X of HSV-1 infected X gal stained anaerobic HeLa cells; (D2a) Representative photomicrograph 10X of HSV-2 infected X gal stained aerobic HeLa cells; (D2b) Representative photomicrograph 10X of HSV-2 infected X gal stained anaerobic HeLa cells

The ability to initiate the replicative cycle under aerobic and anaerobic conditions was confirmed through the detection of HSV entry-stage protein expression (ICP8, ICP27, VP5) by fluorescent microscopy (Figure 2) [12-16]. Control for the presence of cell-associated HSV1/2 were cells secondarily stained with HSV1/2 glycoprotein B mouse monoclonal antibody and 4',6-diamidino-2-phenylindole (DAPI). On Day 1, cells were infected with an MOI of 1, of either HSV-1 or HSV-2, then incubated under aerobic and anaerobic conditions.





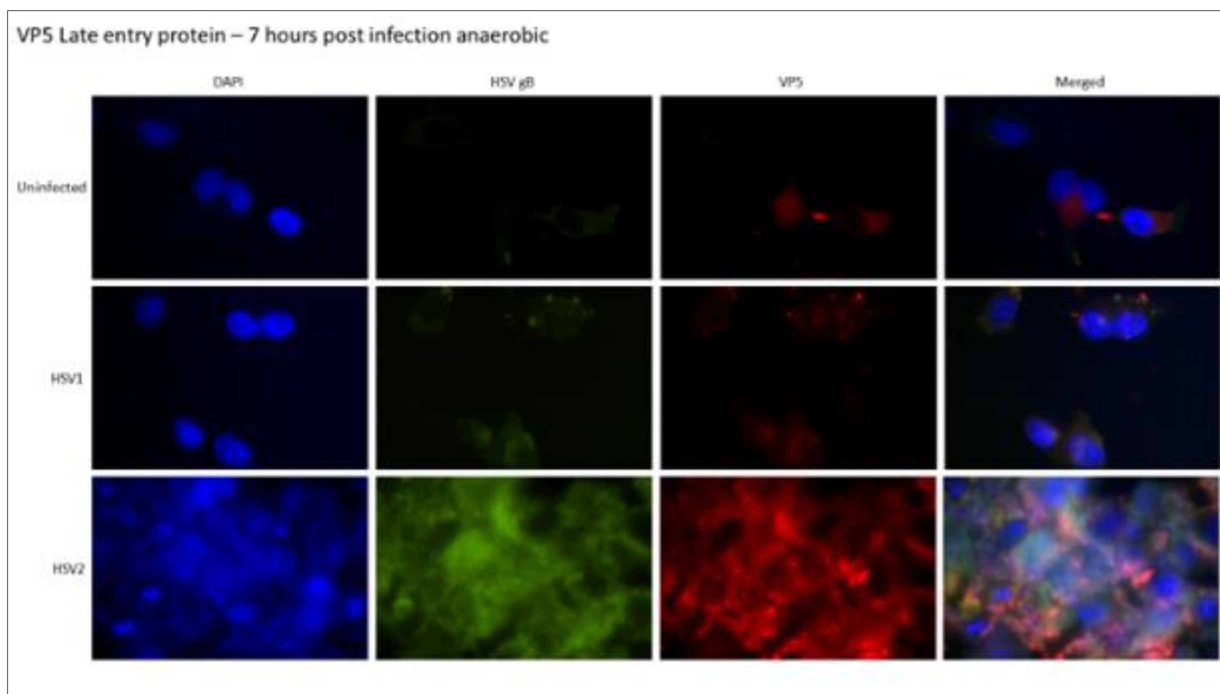


Figure 2 HSV1/2 and entry protein expression in response to incubation in the absence of oxygen. (A) ICP8 Early Entry Protein expression (B) ICP27 Immediate Early Entry Protein; (C) VP5 Late Entry Protein. HeLa cells were infected with HSV-1 (MOI 1) or HSV-2 (MOI 1). After 7h post-infection, cells were fixed and then stained with HSV gB (green) and entry protein (red) antibodies.

Microscopic analysis showed that all three entry proteins were detected for both HSV-1 and HSV-2. The relative levels of protein expressed appeared higher for HSV-2 vs. HSV-1, likely reflective of the difference in level of infection as indicated by ONPG and X-gal staining (Fig 1).

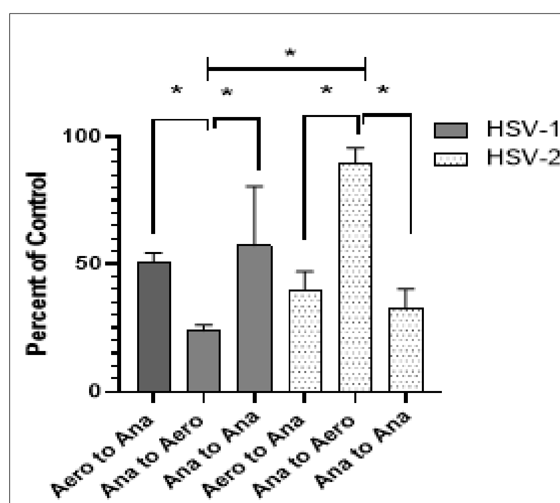


Figure 3 Effect of oxygen on viability of HSV1/2 progeny when tested under homologous and heterologous growth conditions. Percent of control (progeny from HSV-1 and HSV-2 aerobic-to-aerobic incubation conditions) for each condition tested. Aero= aerobic; ana= anaerobic; * = $p \leq 0.05$

To confirm that HSV1/2 was going through a productive replicative cycle in the anaerobic model system, progeny isolated from aerobic and anaerobic conditions were grown under homogenous atmospheric environment, as well as transferred to heterologous oxygenated conditions. Their viability was tested for the ability to cause cell infection, as described above (Figure 3). Interestingly, the infection levels as compared to their control i.e., progeny isolated after aerobic replication cycle and transferred to aerobic cells, differed for HSV-1 and HSV-2. Significantly ($p \leq 0.05$) fewer viable progeny were present from HSV-1 anaerobic cultures that were subsequently transferred to aerobic culture

incubation compared to similarly incubated HSV-2 progeny. HSV-2 progeny from anaerobic cultures tested aerobically had levels of viable progeny similar to the HSV-2 aerobic-aerobic control. Progeny incubated anaerobically, regardless of initial incubation conditions, e.g., anaerobic, or aerobic, were similar for both HSV-1 and HSV-2.

4. Conclusion

Despite the likelihood that HSV infections *in vivo* occur in a low oxygen environment, HSV are typically grown under carbon dioxide enriched aerobic conditions (21% oxygen) *in vitro*, even during antibiotic sensitivity testing. These studies show that HSV1/2 exhibits a productive replicative cycle under anaerobic conditions *in vitro*. Data shows a reduction in the number of HSV-1 infected cells observed under anaerobic conditions when compared to aerobic conditions at the same MOI as compared to HSV-2. Furthermore, the pattern of progeny infection in response to heterologous growth conditions indicates that HSV-2 is more robust in its ability to adapt to shifts in levels of oxygenation. This alteration in its phenotype under oxygen stress conditions may impact pathogenesis and the ability to treat infections, potentially indicating novel targets for developing anti-viral drugs. Further studies characterizing hypoxic effects and oxygen-regulated susceptibility of herpes to anti-viral agents are in progress.

Compliance with ethical standards

Acknowledgments

This work was funded by the Midwestern University Office of Research and Sponsor Programs, the MWU College of Graduate Studies, the Chicago College of Osteopathic Medicine, and supported by the College of Dental Medicine.

Disclosure of conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author's contribution

Balbina J. Plotkin: Project conception, investigation, formal analysis, methodology, writing –original draft. **Ira M. Sigar**: Methodology, data collection, writing, manuscript reviewing. **Amber Kaminski**: Methodology, microscopy, data collection, manuscript reviewing and editing. **Alexis Borrelli:** Data collection, writing- review and editing. **Victoria Zheng**: Data collection, writing- review and editing. **Nicholas Schileru:** Data collection, writing- review and editing. **Michelle Aelion:** Data collection, writing- review and editing. **Brandon Siaj:** Data collection, writing- review and editing.

References

[1] Carreau A, Hafny-Rahbi BE, Matejuk A, Grillon C, Kieda C. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. Journal of Cellular and Molecular Medicine. 2011, 15(6):1239-53.

[2] Biedermann L, Rogler G. The intestinal microbiota: its role in health and disease. European Journal of Pediatrics. 2015, 174(2):151-67.

[3] Ortiz-Prado E, Dunn JF, Vasconez J, Castillo D, Viscor G. Partial pressure of oxygen in the human body: a general review. American journal of blood research. 2019, 9(1):1.

[4] Flück K, Fandrey J. Oxygen sensing in intestinal mucosal inflammation. Pflügers Archiv-European Journal of Physiology. 2016, 468(1):77-84.

[5] Plotkin BJ, Davis JW, Strizzi L, Lee P, Christoffersen-Cebi J, Kacmar J, et al. A method for the long-term cultivation of mammalian cells in the absence of oxygen: Characterization of cell replication, hypoxia-inducible factor expression and reactive oxygen species production. Tissue and Cell. 2018, 50:59-68.

[6] Plotkin BJ, Sigar IM, Swartzendruber JA, Kaminski A, Davis J. Differential expression of cytokines and receptor expression during anoxic growth. BMC Research Notes. 2018, 11(1):406.

[7] Plotkin BJ, Sigar IM, Kaminski A, Konaklieva MI. Putative pathways fueling anaerobic mitochondrial respiration. system. 2022, 13:30-2.

[8] Tiwari V, Shukla SY, Shukla D. A sugar binding protein cyanovirin-N blocks herpes simplex virus type-1 entry and cell fusion. Anti-viral research. 2009, 84(1):67-75.

[9] Tiwari V, Darmani NA, Yue BY, Shukla D. In vitro anti-viral activity of neem (Azardirachta indica L.) bark extract against herpes simplex virus type-1 infection. Phytotherapy Research. 2010, 24(8):1132-40.

[10] Montgomery RI, Warner MS, Lum BJ, Spear PG. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell. 1996, 87(3):427-36.

[11] Plotkin BJ, Sigar IM, Tiwari V, Halkyard S. Herpes Simplex Virus (HSV) Modulation of Staphylococcus aureus and Candida albicans Initiation of HeLa 299 Cell-Associated Biofilm. Current Microbiology. 2016, 72(5):529-37.

[12] Akhtar J, Tiwari V, Oh MJ, Kovacs M, Jani A, Kovacs SK, et al. HVEM and nectin-1 are the major mediators of herpes simplex virus 1 (HSV-1) entry into human conjunctival epithelium. Investigative Ophthalmology & Visual Science. 2008, 49(9):4026-35.

[13] Tiwari V, Liu J, Valyi-Nagy T, Shukla D. Anti-heparan sulfate peptides that block herpes simplex virus infection in vivo. J Biol Chem. 2011, 286:25406-15.

[14] Ali MM, Karasneh GA, Jarding MJ, Tiwari V, Shukla D. A 3-O-sulfated heparan sulfate binding peptide preferentially targets herpes simplex virus 2-infected cells. Journal of virology. 2012, 86(12):6434-43.

[15] Hubbard S, Choudhary S, Maus E, Shukla D, Swenson S, Markland FS, et al. Contortrostatin, a homodimeric disintegrin isolated from snake venom inhibits herpes simplex virus entry and cell fusion. Anti-viral Therapy. 2012, 17(7):1319-26.

[16] Shukla SY, Singh YK, Shukla D. Role of Nectin-1, HVEM, and PILR-alpha in HSV-2 entry into human retinal pigment epithelial cells. Invest Ophthal Vis Sci. 2009, 50(6):2878-87.

[17] Rashad AL, Toffler WL, Wolf N, Thornburg K, Paul Kirk E, Ellis G, et al. Vaginal Po2 in healthy women and in women infected withTrichomonas vaginalis: Potential implications for metronidazole therapy. American Journal of Obstetrics and Gynecology. 1992, 166(2):620-4.

[18] Wald A, Ashley-Morrow R. Serological Testing for Herpes Simplex Virus (HSV)–1 and HSV-2 Infection. Clinical Infectious Diseases. 2002, 35(Supplement_2):S173-S82.

[19] Sucato G, Wald A, Wakabayashi E, Vieira J, Corey L. Evidence of latency and reactivation of both herpes simplex virus (HSV)-1 and HSV-2 in the genital region. Journal of Infectious Diseases. 1998, 177(4):1069-72.

[20] Plotkin BJ, Sigar IM, Swartzendruber JA, Kaminski A. Anaerobic Growth and Maintenance of Mammalian Cell Lines. JoVE. 2018(137):e58049.