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Authors
Turner, Meghan T
Nayak, Shruti
Kuhn, Maggie
et al.

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The effects of dexamethasone and acyclovir on a cell culture model of delayed facial palsy

Meghan Thompson-Turner, M.D.[1], **Shruti Nayak, M.S.**[1], **Maggie Kuhn, M.D.**[2], and **Pamela C. Roehm, M.D., Ph.D.**[3]

[1]Department of Otolaryngology, New York University School of Medicine, New York, NY U.S.A. 10016

[2]Department of Otolaryngology, University of California, Davis, Sacramento, CA 95817

[3]Department of Otolaryngology—Head and Neck Surgery, Temple University School of Medicine, Philadelphia, PA 19140

Abstract

Hypothesis—Pretreatment with antiherpetic medications and steroids decreases likelihood of development of delayed facial paralysis (DFP) following otologic surgery.

Background—Heat-induced reactivation of herpes simplex virus type 1 (HSV1) in geniculate ganglion neurons (GGNs) is thought to cause of DFP after otologic surgery. Antiherpetic medications and dexamethasone are used to treat DFP. Pretreatment with these medications has been proposed to prevent development of DFP.

Methods—Rat GGN cultures were latently infected with HSV1 expressing a lytic protein-GFP chimera. Cultures were divided into pretreatment groups receiving acyclovir (ACV), acyclovir-plus-dexamethasone (ACV+DEX), dexamethasone alone (DEX), or untreated media (control). Following pretreatment, all cultures were heated 43°C for two hours. Cultures were monitored daily for reactivation with fluorescent microscopy. Viral titers were determined from culture media.

Results—Heating cultures to 43°C for two hours leads to HSV1 reactivation and production of infectious virus particles (59% ± 6.8); heating cultures to 41°C showed a more variable frequency of reactivation (60% ± 40); compared with baseline rates of 14.4% ± 5. Cultures pretreated with ACV showed lower reactivation rates (ACV = 3.7%, ACV+DEX = 1.04%) compared to 44% for DEX alone. Viral titers were lowest for cultures treated with ACV or ACV+DEX.

Conclusion—GGN cultures harboring latent HSV1 infection reactivate when exposed to increased temperatures that can occur during otologic surgery. Pretreatment with ACV prior to heat provides prophylaxis against heat-induced HSV reactivation, while DEX alone is associated with higher viral reactivation rates. This study provides evidence supporting the use of prophylactic antivirals for otologic surgeries associated with high rates of DFP.

Corresponding author: Pamela C. Roehm, M.D., Ph.D. Department of Otolaryngology—Head and Neck Surgery Temple University School of Medicine 3400 N. Broad Street, Kresge West 3rd Floor Philadelphia, PA 19140 phone 215-707-3663 fax 215-707-6417 pamela.roehm@tuhs.temple.edu.

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INTRODUCTION

Delayed facial palsy (DFP) is a temporary but potentially devastating complication of otologic, neurotologic, and neurosurgical procedures and temporal bone trauma. DFP is a unilateral peripheral facial paralysis with time of onset 4—14 days following these procedures or traumatic insult to the temporal bone. The paralysis can be either complete or incomplete. Timing to recovery varies but typically full recovery will occur within 9—12 months.

The incidence of DFP following surgical procedures varies greatly based on the type of procedure. Rates range from 1.4% following mastoidectomy up to 35% following acoustic neuroma resection (1-11) (**Fig. 1**). For stapes surgery, the incidence of DFP is 0.2—0.51% (12,13).

There is clinical evidence that DFP is the result of reactivation of latent herpes simplex type I (HSVI) virus in geniculate ganglion neurons, that then causes inflammation and edema of the labyrinthine segment of the facial nerve. HSV1 viral titers increase following DFP; and rates of DFP following pretreatment with famciclovir prior to acoustic neuroma resection drop significantly (7,11,14). During otologic and neurotologic surgeries, increased temperatures (1.4—80°C) can result from use of instruments necessary for these procedures (15-19). Some authors have hypothesized that the excess heat generated by instruments intraoperatively may cause DFP (17,18,20-22).

Although facial function typically recovers completely over the course of months, DFP can be devastating to patients. DFP is typically treated with high-dose steroids, antiherpetic medications, or a combination of both medications (1-13). This is based on extension of treatment guidelines for Bell's palsy to DFP, as there is no high level clinical evidence regarding treatment of DFP following onset of facial paresis.

In this study, we have developed a model system for the study of DFP using cultured geniculate ganglion neurons (GGNs) latently infected with HSV1. Heating the neurons consistently yields HSV1 reactivation and infectious virus. Using this system, we have studied the effects of pretreatment of latently infected GGNs with acyclovir and prednisone on HSV1 reactivation rates. We have also measured effects of viral reactivation on neuronal morphology. Finally, we have measured the production of infectious virus following heat treatment of latently infected GGNs as well as for each of the pretreatment regimens.

METHODS

GGN Harvest, Purification, and Cell Culture

GGN neurons were harvested from 5-day old Sprague-Dawley rat pups and cultured as dissociated cultures as previously described on 96-well plates (BD Falcon) (23). All experimental methods involving animals were approved by the IACUC committee of the NYU School of Medicine (protocol # 120207-02). Cultures were monitored daily with light microscopy for neuronal health and signs of infection.

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HSV1 infection

The HSV1 strain used in these experiments was the HSV1 Patton strain with a US11 green fluorescent protein chimera (HSV1/GFP) (24). Dilutions of the stock solution were made for GGN inoculation by calculating the average number of neurons per well.

Induction of Primary Lytic HSV1 Infection

On day *in vitro* (DIV) 4, GGN cultures were infected with HSV1/GFP at an MOI of 0.5 for 1.5 hours at 37°C. The virus was removed and replaced with fresh cell culture medium consisting of Neurobasal medium (Gibco, Grand Island, NY U.S.A) supplemented with B27 additives (Gibco), 5% fetal bovine serum (Gibco), 20 μmol/L Z-VAD-FMK (ZVAD; Calbiochem, Darmstadt, Germany) , and 0.0003% ofloxacin (Daiichi Pharmaceutical Corp., Tokyo, Japan). GFP+ neurons were detected the day after infection by fluorescent microscopy. Lytic infection was established in at least one well per experimental group as a positive control for successful inoculation and as check for activity of the viral solution.

Induction of Latent HSV1 Infection

On DIV2 selected wells were treated with 100 μmol/L acyclovir (ACV, Calbiochem) in order to establish latent infection, as described in (23). On DIV4, these neurons were infected with HSV1/GFP in the presence of ACV. Following infection, cultures were maintained in the presence of ACV for 4 more days, until DIV 8. Latently infected cultures were inspected daily for neuronal health and signs of lytic HSV1 infection by light microscopy. Latently infected cultures were also checked daily using fluorescent microscopy for GFP+ cells. At DIV 8, the ACV was removed from culture media unless otherwise indicated.

Heat treatment of latently infected GGN cultures

GGN cultures were latently infected as noted above. On DIV 8, all wells were checked for GFP+ neurons using fluorescent microscopy. Any latently infected wells exhibiting GFP positivity were excluded from the remainder of the experiments.

Latently infected cultures were pretreated or mock pretreated as shown in **Figure 2**. The ACV group was kept in cell culture media including 100 μmol/L ACV (Calbiochem). The DEX group was given cell culture media supplemented with 1 μg/mL dexamethasone (Sigma Chemical Co., St. Louis, MO, USA). The ACV+DEX treated neurons were treated with 100 μmol/LACV and 1 μg/mL dexamethasone. Culture wells in the control group had fresh cell culture media replaced into the wells. No ACV or DEX was present in control media.

The following day (DIV 9) the cultures were heated at either 41°C or 43°C in a cell culture incubator for 2 hours. Cultures were then replaced into a 37°C cell culture incubator. Heat-control cultures were maintained at 37°C throughout these experiments. CO_2 concentrations were maintained at 5% throughout the experiments.

Cultures were monitored daily for development of GFP+ neurons using fluorescent microscopy. Photomicrographs were taken to document GFP+ of cultures.

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Initial experiments to determine the ideal temperature to induce HSV1 reactivation were repeated 3 times. Experiments to determine the results of treatment regimens versus control were repeated 12 times.

Reverse transcription polymerase chain reaction

Total RNA was isolated from GGN cultures that were lytically infected at 37°C, latently infected and maintained at 37°C, or heated to 43°C for two hours as described above. RNA was collected from cells in these wells using RNAzol RT per the manufacturer's instructions (Molecular Research Center, Cincinnati, OH U.S.A.). Samples taken from 3 wells were combined. Complementary DNA was produced from each total RNA sample, and reverse transcription polymerase chain reaction (RT-PCR) was performed using the Qiagen OneStep RT-PCR kit (Qiagen, Valencia CA U.S.A.) supplemented with RNAsin (Sigma), on an Eppendorf Mastercycler epGradientS (Eppendorf AG, Hamburg, Germany). Primers used include those identifying the LAT transcript, ICP27, and GAPDH, as described previously (25). H_2O controls were performed with each primer set to determine whether or not contamination had occurred. RT-PCR reaction products were run in separate wells on 1.25% agarose TAE-ethidium bromide gels.

Viral titer determination

The media from control, ACV, ACV + DEX, and DEX pretreated cultures was collected at DIV 14. Media from lytically infected wells was collected at DIV 8. Viral titers were determined by plaque assay on confluent Vero cell cultures. Each media sample was titered in duplicate. Titers of viral stocks were performed in similar fashion.

Statistical analysis

Comparisons of effect of heat treatments versus baseline 37°C reactivation and between different pretreatment regimens were performed using Fisher's exact test to calculate 2 sided *p*-values utilizing the Microsoft Research site Fisher exact test calculator http://research.microsoft.com/en-us/um/redmond/projects/mscompbio/fisherexacttest/. Comparisons of infectious viral yield determined by titer of media from latently infected cultures were performed using Mann-Whitney U-test and Wilcoxon matched-pairs signed rank calculations performed on GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). *p* values less than 0.05 were considered significant.

RESULTS

Induction of HSV1 reactivation with heat

GGN cultures were latently infected with HSV1 as described above. On DIV 8, ACV was withdrawn from the culture media. GGN cultures were heated to either 41°C or 43°C for 2 hours in a 5% CO_2 cell culture incubator. Following this heat treatment, cultures were returned to a 37°C 5% CO_2 cell culture incubator. Cultures were assessed daily for the presence of GFP+ cells using fluorescent microscopy. Results are shown in **Figures 3**. Rates of HSV1 reactivation were increased at both 41°C and 43°C (60 ± 40% and 59 ± 6.8% respectively) compared with cultures that were never heated above 37°C (14 ± 5%; $p <$

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0.05). Due to the greater variability of induction of reactivation at 41°C, 43°C was the temperature used in the rest of the experiments.

HSV1 latency in cultures maintained at 37°C under baseline conditions and lytic reactivation in cultures heated to 43°C was confirmed using RT-PCR of total RNA obtained from cultures (**Fig. 4**). During latency, the only HSV1 transcript present should be the latency-associated transcript (LAT), as seen for the latently infected GGN cultures maintained at 37°C. In cultures with lytic, productive infections, such as the lytic control and 43°C-treated cultures, other viral RNAs, such as icp27, are transcribed in addition to LAT. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a cellular gene, was used as a marker of total RNA content within the RT-PCR reactions (**Fig. 4**).

Pretreatment of latently infected GGN cultures with acyclovir and dexamethasone

At DIV 8, latently infected GGN cultures were pretreated with either acyclovir (ACV), dexamethasone (DEX), or a combination of both (ACV+DEX). Control cultures underwent culture media substitution without addition of DEX or ACV. One day later (DIV 9), cultures were placed into a 43°C / 5% CO_2 cell culture incubator for 2 hours. The cultures were then returned to the 37°C / 5% CO2 incubator per the protocol shown in **Figure 2**. Cultures were assessed daily for GFP+ cells using fluorescent microscopy. Results are shown in **Figures 5 and 6**.

HSV1 reactivation was severely limited following pretreatment with acyclovir. For control cultures, which received media without added acyclovir or dexamethasone and subsequently were heated to 43°C, viral reactivation occurred in 55 ± 7% of wells (**Fig. 6**). Reactivation rates in the presence of ACV alone were 3.7 ± 2.8%, which was significantly lower than cultures heated to 43°C in the absence of ACV ($p < 0.05$). Comparison of the ACV and ACV+DEX treated cultures revealed reactivation rates that were not significantly different (ACV + DEX reactivation rate, 1.04 ± 1%; $p = 0.369$). In comparison, following pretreatment with dexamethasone alone (DEX), HSV1 reactivation was seen in 42 ± 10% of latently infected wells. This was not significantly different than the percentage of reactivation seen in the control 43°C heated wells ($p = 0.157$).

Media from pretreated cultures was examined for the presence of infectious virus using plaque assays on Vero cells (**Fig. 7**). Media was titered from at least 10 samples per condition in duplicate. Latently infected cultures maintained at 37°C contained no infectious viral particles. Media from latently infected cultures maintained at 37°C and treated with trichostatin A (TSA) at DIV 14 (positive 37°C HSV1 reactivation control) had titers which did not differ significantly from titers of media from lytically infected GGN cultures (1.75×10^4 pfu/ml v. 1.925×10^4 pfu/ml, $p = 0.4988$). Cultures heated to 43°C with control media demonstrated a slightly lower average titer compared with TSA/37°C and lytic controls (1.02×10^3 pfu/ml). The 43°C control titer was significantly different than the positive control ($p < 0.05$) but also significantly greater than the baseline 37°C maintained culture titer (0 pfu/ml over multiple replications, $p < 0.005$). All cultures undergoing pretreatment regimens prior to heating to 43°C had lower levels of infectious virus within their media. Both the ACV and ACV+DEX conditions had no titerable viral particles within the media recovered. The DEX only condition had an extremely low average titer of infectious viral

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particles (365 + 251 pfu/ml); this level was significantly lower than that found in the 43°C, nonpretreated group ($p = 0.0029$). Titers in the DEX only condition varied greatly, with a range of 0—1 × 10^3 pfu/mL; however, most of the DEX only culture wells sampled had no titerable virus (7/10 wells).

DISCUSSION

DFP can be a devastating complication for patients following otologic and neurotologic procedures. Despite these consequences, there is limited medical literature regarding the appropriate prevention and treatment of this disorder. We have developed a model for surgically-induced DFP using cultured GGNs latently infected with HSV1 and subjected to increased levels of heat. Using this model, we have demonstrated that GGN cultures latently infected with HSV1 undergo high levels of viral reactivation after being heated to 41 or 43°C, with production of infectious virus. Pre- and continued treatment with acyclovir can block heat-induced reactivation, while pretreatment with dexamethasone has no effect on rates of HSV1 reactivation in cultured GGNs. No infectious virus is produced in cultures pretreated with ACV or ACV + DEX, and substantially lower levels of infectious virus are produced following pretreatment with DEX alone.

There is substantial clinical evidence demonstrating that cases of DFP occurring more than a few days after surgery or trauma are often the result of reactivation of a neurotrophic virus. Two herpesviruses, HSV1 and varicella-zoster virus (VZV), have been implicated as viruses that may cause DFP. Serologic evidence demonstrates that titers against HSV1 increase during DFP following both stapes surgery and acoustic neuroma resection (7,14). Anti-VZV titers also increase following acoustic neuroma removal (7). There are two case reports of a delayed facial palsy resulting from VZV reactivation following surgical manipulation, with onset of DFP following middle ear surgery (26) and acoustic neuroma resection (27). In the case following acoustic neuroma resection, DFP was followed by subsequent appearance of vesicles in the trigeminal V_2 distribution (27). Brackmann et al (2008) found pretreating patients with famciclovir, and antiherpetic medication similar to acyclovir, for 3 days prior to and 5 days following acoustic neuroma resection significantly decreased rates of DFP from 25.3% to 20.6% (11).

Several authors have suggested that DFP may be related to exposure of the geniculate ganglion or surrounding areas of the facial nerve to elevated temperatures (17,18,20-22). In other model systems, HSV1 reactivates following heat treatment. Sawtell and Thompson (1992) inoculated mice with a modified HSV1 strain and one month later heated the animals to 43°C for 3 minutes. Trigeminal ganglia harvested from the heated animals yielded infectious viral particles (28). In another *ex vivo* model of HSV1 reactivation, trigeminal ganglia were harvested from mice that were previously inoculated with HSV1 strain F. Cultures of the trigeminal ganglion neurons from these animals were heated to 43°C for 3 hours demonstrated HSV1 reactivation (29). Hunsperger and Wilcox demonstrated that heating latently infected cultured dorsal root ganglion cultures for 2 hours produced infectious viral particles (30) Thus, there is experimental evidence from multiple model systems supporting the reactivation of HSV1 in latently infected neurons following treatment with heat.

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Use of equipment routinely used in otologic and neurotologic procedures can increase temperatures in the fallopian canal or exposed facial nerve substantially. Tissue temperatures can rise 4°C during use of a high-speed otologic drill, even when performed by an experience neurotologist and with use of copious irrigation (15,17). CO_2 and argon lasers can increase temperatures of surrounding bone and tissues 9-15°C (16,18). The operating microscope can transfer significant heat energy to illuminated tissues, up to 80°C in the case of xenon microscopes (19). These potential heat sources can transfer heat more efficiently in the presence of a dehiscent facial nerve. Thus there are multiple potential mechanisms by which sufficient thermal energy could be transferred to the geniculate ganglion and facial nerve during otologic and neurotologic procedures to induce HSV1 reactivation.

Modeling a clinical entity on a cellular and molecular level can greatly expand clinical understanding, prevention or avoidance, and treatment options that might not previously have been considered based on clinical studies alone. Studies of DFP in humans are limited its low incidence in all but certain neurotologic procedures, complicated by the presence of a functional immune system, and concerns regarding worsening of an already debilitating condition. Whole animal studies of HSV1 reactivation and DFP in particular are also limited, by long duration of the experiments, relative inaccessibility of the neurons in question (in the case of facial palsy), and significant differences between animals and humans in terms of establishment of latent HSV1 infection and reactivation.

The cell culture system used within this study is a model system, and as such has limitations. It utilizes rat neurons rather than human. In order to induce latent HSV1 infection in these neurons, they are treated with ACV around the time of HSV1 infection. Latent infection with HSV1 can also be induced in these cultures at very low multiplicities of infection; however, this yields very low rates of latent infection and reactivation, as would be expected.

Our cell culture system does have several significant advantages over human and whole animal studies of HSV1 reactivation, particularly with respect to modeling processes involved in DFP. Our system allows experiments to be completed within a few weeks' time, so multiple repetitions of each experiment can readily be performed. Our system allows for strict control of more experimental variables than those in whole animal experiments, and more readily allows for molecular and cellular level analyses than whole animal or human experiments. Fewer animals are required than in whole animal experiments, and the model system does not face the considerable hurdles of interpretation of data in the setting of a whole organism with intact immune function. Finally this cell culture system does not have limitations of the standard of care or side-effect profiles of treatments. Using cell culture systems of HSV1 reactivation, both we and others have found results similar to those seen in whole animal and human studies, and have been able to more rapidly build on those results.

Typically, DFP is treated with either high-dose steroids, antiherpetic medications, or both (1-13). Due to the low incidence of DFP, treatment for DFP is not supported by level I clinical evidence, but instead has been extrapolated from accepted therapy for Bell's palsy. Prophylactic treatment with famciclovir prior to acoustic neuroma resection in one prospective trial significantly decreased the incidence of DFP compared with untreated

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controls by 5% (11). In that study, the dosage and timing famciclovir were chosen to limit potential side effects, and were not optimized for maximal prevention of DFP. Here we have presented findings from our model system that support prophylactic treatment for HSV1 reactivation with the possible addition of steroid therapy prior to surgical procedures with a high incidence of DFP.

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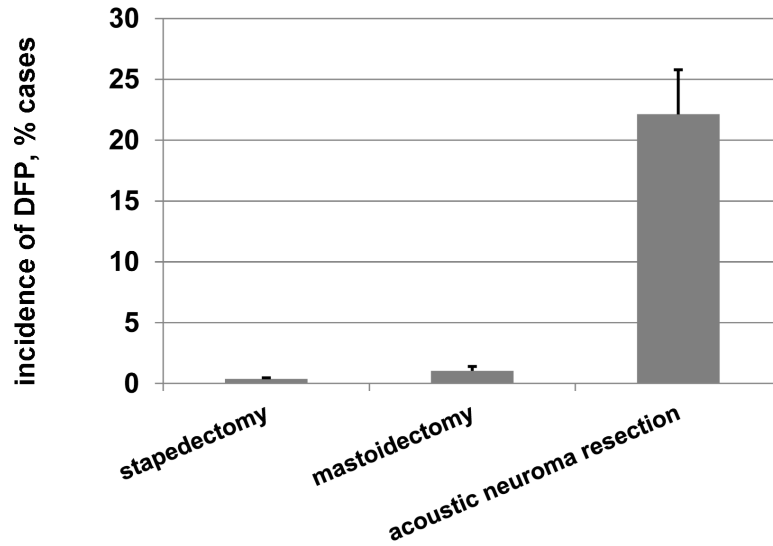

Figure 1. Percentages of patients with delayed facial palsy following otologic procedures

Average percentages of patients with delayed facial palsy following otologic (mastoidectomy or cochlear implantation (1,9), stapedectomy / stapedotomy (12,13,31) and acoustic neuroma excision (by any approach) (2,3,5,6,8,10,11) were extracted from the medical literature. Where more than one paper measured the percentage following a particular procedure, those numbers were combined in a weighted average. Error bars measure the standard error of the mean.

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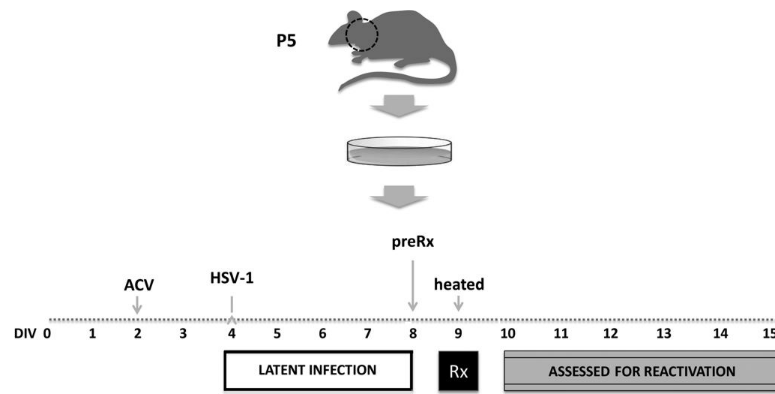

Figure 2. Experimental course for HSV1 latency and pretreatment experiments

GGN neurons were harvested from postnatal day 5 rats and cultured. On DIV 2, cultures were treated with ACV. Cultures were infected with HSV1 on DIV4 in the presence of ACV and maintained in ACV for 4 more days. On DIV 8, cultures were pretreated with either ACV, DEX, ACV+DEX, or control. On DIV 9, cultures were heated to 43°C for 2 hours. Subsequently cultures were maintained in normal cell culture conditions (37°C/ 5% CO_2) and monitored daily for evidence of HSV1 reactivation.

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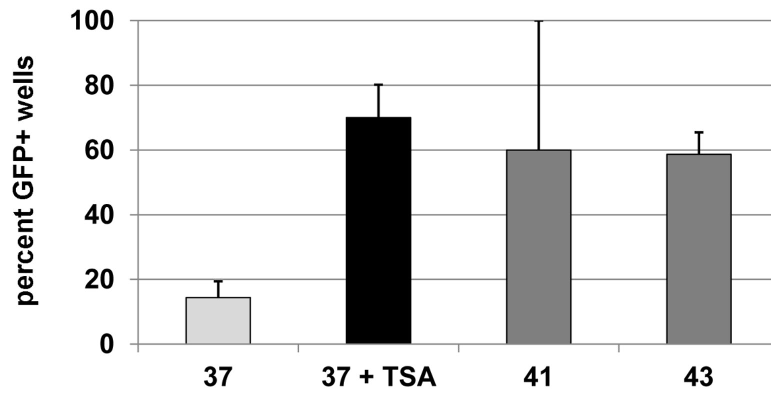

Figure 3. HSV1 reactivation following heat treatment

Latently infected GGN cultures were heated to 41 or 43°C for 2 hours. Reactivation of HSV1 was monitored by GFP fluorescence. Percent reactivation was compared with unheated controls. Error bars measure standard error of the mean.

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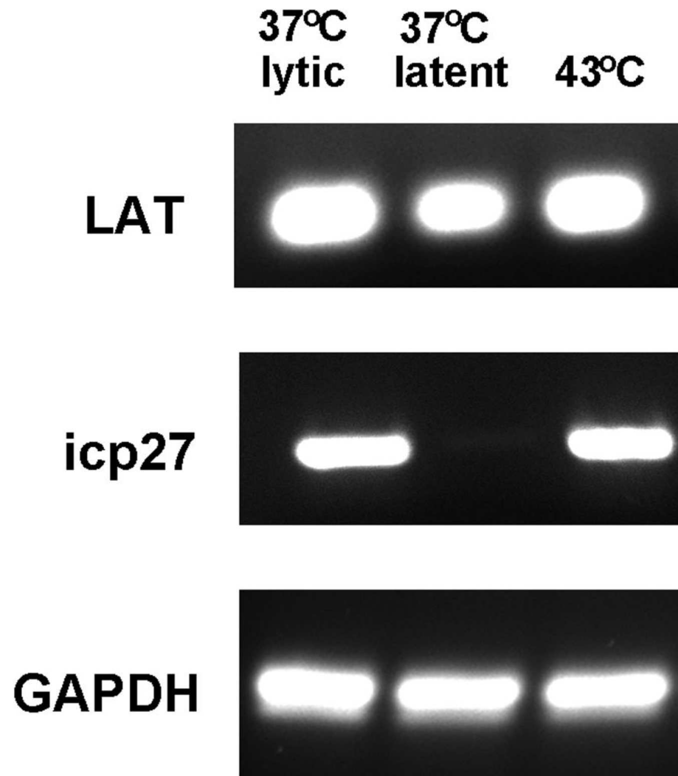

Figure 4. RT-PCR of lytic, latently infected, and latently infected heated cultures

GGN cultures were either lytically or latently infected as described. Latently infected cultures were either maintained at 37°C or heated to 43°C for 2 hours. Total RNA from cultures was harvested at DIV 8 (lytically infected cultures) or DIV 14 (latently infected cultures). cDNA was generated from total RNA and then specific targets were amplified using PCR. The latency associated transcript, LAT, was the only viral transcript present in latently infected cultures maintained at 37°C. Other viral transcripts, including icp27, could be amplified from lytic and latently infected, 43°C heated cultures, consistent with a productive viral infection. GAPDH, a cellular gene, was used as a marker of total RNA levels in the reactions.

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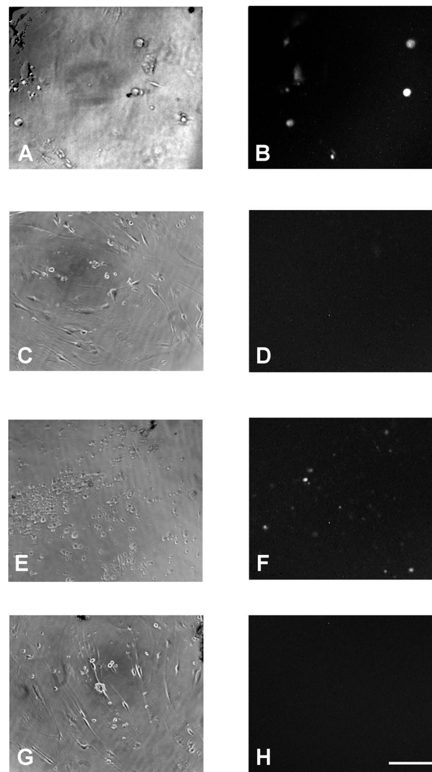

Figure 5. Photomicrographs of the effects of acyclovir and dexamethasone on heat-induced HSV1 reactivation

GGN cultures latently injected with HSV1 were not pretreated (control) (A,B) or pretreated with acyclovir (ACV) (C,D), dexamethasone (DEX) (E,F), or ACV + DEX (G,H) for 24 hours on DIV 9. 24 hours later these cultures were heated in a cell culture incubator to 43°C for 2 hours and then returned to a 37°C incubator for the remainder of the experiments. Cultures were monitored for reactivation daily by light microscopy for cellular morphology and fluorescent microscopy for GFP. Both control heated cultures and DEX treated cultures demonstrated cellular changes consistent with HSV1 reactivation and GFP fluorescence; these changes were not seen in cultures treated with ACV or ACV + DEX. Size bar = 200 micrometers.

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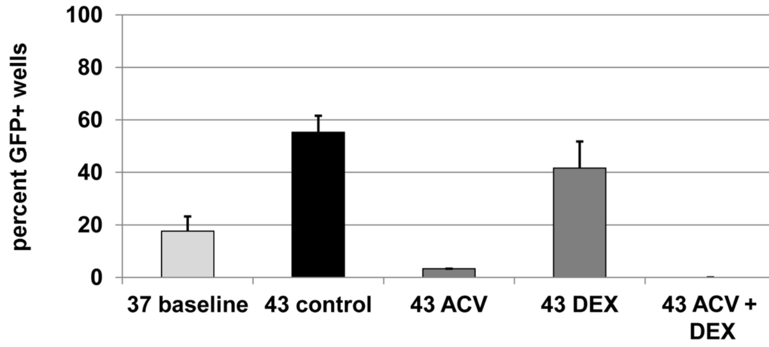

Figure 6. Effects of acyclovir and dexamethasone on heat-induced HSV1 reactivation

Latently infected GGN cultures were pretreated with either acyclovir (ACV), dexamethasone (DEX) or ACV+ DEX for 24 hours and then heated to 43°C for 2 hours. Rates of HSV1 reactivation were compared with controls (43 control) that were not pretreated (normal media change) prior to heating to 43°C. Also shown are baseline percentages of reactivation in cultures maintained at 37°C (37 baseline). Both 43°C controls and DEX treated cultures showed increased rates of reactivation compared with baseline cultures maintained at 37°C. There was no significant difference in the rate of reactivation of 43°C controls and DEX treated wells. ACV and ACV + DEX treated cultures had significantly lower percentages of reactivation than baseline 37°C controls. Error bars measure standard error of the mean.

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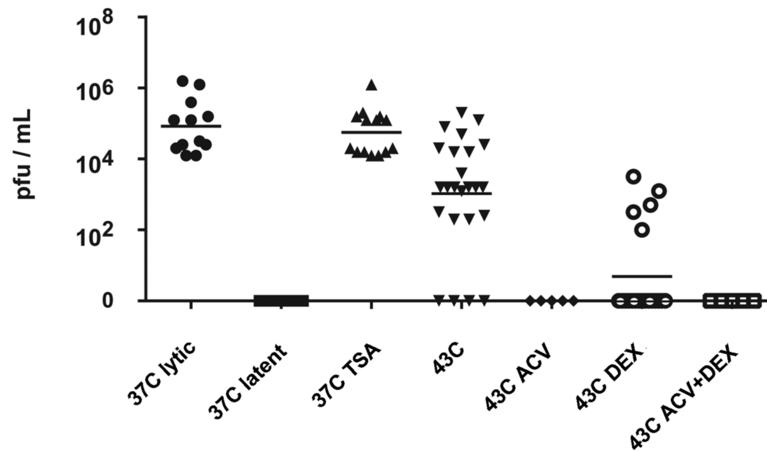

Figure 7. Titers of media from 43°C-heated cultures

Media from cultures lytically infected at 37°C (37C lytic); latently infected and maintained at 37°C (37C latent); latently infected, maintained at 37°C and treated with TSA (37C TSA) were compared with cultures that were latently infected and then heated to 43°C at DIV 10 without pretreatment (43C control); or latently infected, pretreated with one of 3 regimens (43C ACV, 43C DEX, or 43C ACV + DEX) and heated to 43°C 24 hours later. Titers were measured in plaque-forming units per milliliter (pfu/mL). Lytically infected and TSA treated cultures showed equivalent titers of infectious virus. 43C control cultures showed a significantly higher titer than latently infected cultures maintained at 37°C. 43C ACV and 43C ACV+DEX cultures had no measureable infectious virus. 43C DEX cultures had very low average titers, but a few cultures had titers comparable to those seen in 43C control cultures. bar = mean.