·Basic Research·

Evaluation of the effects of acyclovir and/or human amniotic membrane on herpes virus culture and quantitative virus inactivity by real-time polymerase chain reaction

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Received: 2013-06-24 Accepted: 2013-09-13

Abstract

• AIM: To investigate the permeability of amniotic membrane in herpes virus cell culture to acyclovir with real time polymerase chain reaction (RT-PCR).

• METHODS: Madin-Darby Bovine Kidney (MDBK) cell culture and Bovine Herpes Virus (BHV1) type 1 were used in the study. Cell cultures were grouped into two on the basis of herpes virus inoculation. Each group was sub-grouped into three. Amniotic membrane (V-HAM), acyclovir (V-A), and amniotic membrane and acyclovir (V-HAM-A) were applied to these subgroup cultures, respectively. After the application of the membrane and the drug, the cultures were evaluated at 24 and 48h for cytopathic effect positive (CPE+) with a tissue culture microscope. In the CPE (+) samples, the DNA was extracted for viral DNA analysis by RT-PCR.

• RESULTS: In control cultures without herpes virus CPE was not detected. Besides, amniotic membrane and acyclovir did not have cytotoxic effect on cell cultures. CPE were detected in Bovine Herpesvirus type -1 inoculated cell cultures after amniotic membrane and/or acyclovir application. DNA analysis with RT-PCR indicated that Cycle threshold (Ct) values were lower in the BHV1 and membrane applied group (amniotic membrane group < acyclovir group < membrane and acyclovir group). This showed that membrane did not have antiviral effect. The membrane and acyclovir cell culture groups with high Ct values indicated that membrane was permeable and had a low barrier effect to drug.

• CONCLUSION: In our *in-vitro* study, we found that amniotic membrane, which can be used in the treatment of corneal diseases, did not have antiviral effect. Besides, we detected that amniotic membrane was permeable to acyclovir in BHV-1 inoculated MDBK cell culture. However, more studies are necessary to investigate the quantitative effects of amniotic membrane and acyclovir.

• KEYWORDS: acyclovir; amniotic membrane; herpes simplex virus
DOI:10.3980/j.issn.2222-3959.2014.04.07

Kantarci FA, Faraji AR, Ozkul A, Akata F. Evaluation of the effects of acyclovir and/or human amniotic membrane on herpes virus culture and quantitative virus inactivity by real-time polymerase chain reaction. *Int J Ophthalmol* 2014;7(4):626-631

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) infection is one of the most common causes of corneal blindness [1-3]. HSV-1 infection is usually identified based on clinical findings. On the other hand, the close relationship between clinical and viral laboratory studies can help to identify suspicious cases. In recent years, the development of molecular diagnostic methods, such as the highly sensitive polymerase chain reaction (PCR) and real-time PCR (RT-PCR), has facilitated early diagnosis and treatment decisions. These methods are widely used because of their high sensitivity for the diagnosis of HSV-1, since they can be used to evaluate more than one specimen at the same time and they provide more rapid results [4-6].

Ocular infection caused by HSV can be treated with various antiviral drugs, such as acyclovir, and topical corticosteroids can be applied when needed [7]. Human amniotic membrane (HAM) is increasingly used in the field of ophthalmology. For severe herpetic corneal infections, HAM can be applied as an alternative to keratoplasty, when medical treatment is unsuccessful, or it can be applied in combination with medical treatment [8,9].

In this study, after the inoculation of Madin Darby Bovine Kidney (MDBK) cell cultures with Bovine Herpes Virus type 1 (BHV-1) and following the application of acyclovir and/or human amniotic membrane, the viral load was analysed by RT-PCR. The effects of acyclovir and the amniotic membrane on the cell and virus cultures were evaluated.

MATERIALS AND METHODS

The BHV-1 (Cooper) reference strains and MDBK cell cultures, which are in the inventory of Ankara University Faculty of Veterinary Medicine Department of Virology, were used to investigate the effectiveness of acyclovir and amniotic membrane *in vitro* by RT-PCR. BHV-1 and HSV-1, which is a member of the alphaherpesvirinae subfamily, are similar viruses. They have the same genomic and biological properties. BHV-1 causes similar infections to HSV-1 in animals [10].

Arrangement of Working Groups The amniotic membrane used in this study was obtained from the placentae of the pregnant donors who were negative for hepatitis virus types B-C, HIV, and syphilis during elective caesarean deliveries under sterile conditions in Gazi University Medical Faculty Hospital Labor&Delivery. The placenta was cleaned of fatal waste and blood clots with a balanced salt solution containing penicillin (50 μm/mL) and neomycin (100 μm/mL). The amnion and chorion were separated from each other by simple blunt dissection, and then the chorion section was removed. The resulting amniotic membrane was spread with the epithelial surface facing up onto sterile nitrocellulose paper, and the stromal matrix adhered to the surface of the paper. This sterile nitrocellulose paper was cut into 5 cm pieces and stored at -80℃ in a petri dish containing corneal storage solutions and glycerol at a ratio of 1:1. This preparation was heated at room temperature for 10min to thaw immediately before the study.

For the MDBK cells, Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal calf serum (FCS) was used as the growth medium, and DMEM containing 2% FCS was used as the storage medium. To prepare 500 mL of DMEM medium, 50 mL FCS was added to 10 mL streptomycin-penicillin solution for the growth medium, and 10 mL FCS was added to 10 mL streptomycin-penicillin solution for the storage medium.

MDBK cell cultures that had been previously prepared and stored frozen at -80℃ and permanently passaged were quickly thawed at 37℃ in a water bath. After being thawed, the MDBK cell cultures were placed into media that were suitable for their cell line. The cells were stored in an incubator until they produced a single layer. To eliminate dead cells in the culture, medium was replaced with 10% FCS. Continuous cultivation of the cell cultures was carried out in 6-well culture plates, and the density of the cells was 100 000 cells per 1 mL. After cultivation of the cell cultures, 3 mL 1000 mL DMEM (Dulbecco's Modified Eagle Medium) and CMC (Carboxymethylcellulose) were added to each well as the growth medium.

In this study, we preferred a non-specific adsorption method to inoculate the virus into the cell cultures because of the high BHV titers. The 6-well culture plates were divided into two groups. In the first group, BHV-1 (DKID50=10^{-6}) was cultivated in three wells. We used a 1/100 dilution of each bottle and a non-specific adsorption method.

A. HAM was placed to cover the growth medium (V-HAM).

B. After placing the HAM, 3% acyclovir was applied to the membrane (V-HAM-A).

C. Acyclovir was applied only for the cell and virus cultivation (V-A).

To evaluate the effects of the amniotic membrane and acyclovir on cells, virus cultivation was not performed in the second group, which contains MDBK cells only. Three wells of the 6-well culture plates from the second group were prepared as follows:

A. HAM was placed to cover the cell culture medium (C-HAM).

B. After placing the cell culture medium on the membrane, 3% acyclovir was applied (C-HAM-A).

C. The drug was applied only to the cell culture medium (C-A).

After the applications, the cell culture petri dishes were incubated with 5% CO_2 at 37℃ for 48h. At 24 and 48h after the inoculation, all cultures were checked with a tissue culture microscope for cytopathic effects (CPE). DNA extraction was performed for cells that showed signs of CPE as well as cells from the other wells. The effects of the amniotic membrane and acyclovir on the viruses and cells were evaluated followed by measurement of the viral load measurement by RT-PCR.

Real-Time Polymerase Chain Reaction In this study, a "Rotor-Gene 6000 (Carbett Research, Australia)" real-time device and TaqMan probes were used according to the "Fluorogenic 5 nuclease method" for the qualitative detection of BHV-1. For diagnosis of the herpes virus, RT-PCR, Black Hole Quencher-1 and Quantitect Virus Kit (Qiagen, Germany) reagents and BHV-1 primers and probes were used. The BHV-1 primers and probes were designed specifically for this study. CLCBio v5. software (Denmark) was used.

Primer and probe indexes:

Primer 1: 5'-AGCTCCGGTTCTACGACATTG-3'

Primer 2: 5'-CCCAGGCCCTGAAAGAAGTTG-3'

Probe: FAM-CGTGGTCAAGACGGACGGCAATA-BHQ1

This primer pair amplified a 90nt region of the BHV-1 glycoprotein B gene. The reaction was performed with a total of 25 μL volume. The composition of the amplification reaction was as follows (Table 1).

For pre-amplification, after the denaturation at 95℃ for 4min, the specimens were processed 45 times with a 2-digit heat cycle (95℃ for 1s and 55℃ for 60s). Fluorescence detection was performed automatically at 55℃ for the denaturation and elongation steps of each cycle of the process.

An amplification graph was used to gather the information needed for the quantitative measurement of DNA or RNA. The Ct (Cycle threshold) is an important parameter for quantitation and indicates the cycle number at which a fluorescent threshold is reached and fluorescence emitted. Compatible exponential growth of fluorescence increases during the log-linear phase of the PCR [11,12].

RT-PCR analysis of the virus specimens were performed for optimisation at dilutions of 10^{-1}, 10^{-2} and 10^{-3} and a pure virus control group. The Ct values of the specimens increased in proportion to the concentration of DNA (Figure 1). Fluorescence propagation of the samples decreased according to the concentration in the exponential phase.

A standard curve was drawn by using the known concentration of the diluted standards in series, and the exact quantitation was performed. The standard curve revealed a linear relationship between the Ct values and the viral DNA load. The linear relationship between the Ct values and the concentration was confirmed in the standard curve graph (Figure 2, Table 2).

Additionally, the brightness of the amplified band in an agarose gel increased from right to left when the BHV-1 DNA concentration increased (Figure 3).

Table 1 RT-PCR mixture reagents and compounds

Componenst of reaction	Volume (μL)
DNA	3
10xReaction Tampon	2.5
$MgCl_2$	2.5
dNTP (10Mm)	0.5
Primary1	1
Primary2	1
Probe	0.8
Hotsart Maxima Taq	0.3
DNA Pol.	
Sterile distiled water	13.4
Total	25

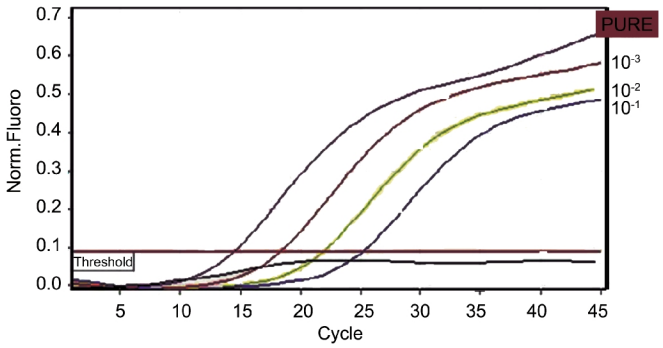

Figure 1 The amplification graph of RT–PCR analysis at 10^{-1}, 10^{-2} , 10^{-3} dilutions and pure virus concentrations.

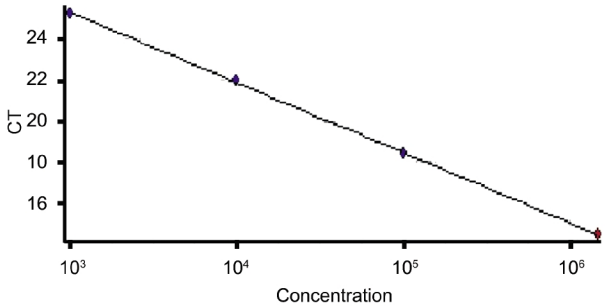

Figure 2 Dilution of the virus concentration for the RT–PCR analysis to generate a standard curve graph.

RESULTS

This study was conducted at the Ankara University Faculty of Veterinary Medicine, Department of Virology, and the experiments were repeated six times. The results of the first three experiments were similar; at 24 and 48h after plating, the DNA was extracted from the cells of groups I and II and evaluated by RT-PCR.

After 24 and 48h, using a tissue culture microscope, the following observations were made for the BHV-1-infected MDBK cells;

A. Only HAM was added to the petri dish, and CPE (+) (V-HAM) was observed (Figure 4).

B. Three percent acyclovir was applied to the HAM and membrane, and CPE (+) (V-HAM-A) was observed (Figure 5).

C. Only 3% acyclovir was applied to the petri dish (+), and CPE (+) was observed (V-A) (Figure 6).

When the CPE (+) cell cultures were investigated with tissue culture microscope, it was evident that the cytotoxicity observed in 24h was increased in 48h and that MDB cells in the background was decreased (Figures 4-6).

The viral load in 3 wells of the 6-well culture plates that were observed separately for CPE (+), were calculated using RT-PCR followed by extraction of the viral DNA from these cells. We evaluated the ability of acyclovir to permeate across the amniotic membrane, the antiviral effect of the amniotic membrane and the effectiveness of acyclovir.

According to the Ct values reported in the graph shown above, the order of the treatment effectiveness was as follows: V-HAM＜V-A＜V-HAM-A (Figure 7). All of the remaining samples (excluding the positive controls) were

Table 2 A graph of the numerical values of the Ct and approximate DNA viral load

Color	Name	Type	Ct	Added concentrations (copy/mL)	Calculated concentrations (copy/mL)	Vary (%)
Red	1	Standard	18.42	1.00E+05	1.03E+05	3.3%
Yellow	2	Standard	21.98	1.00E+04	9.37 E+04	6.3%
Blue	3	Standard	25.24	1.00E+03	1.03E+03	3.3%
Purple	PC	Positive control	14.50		1.47E+06	
Black	NC	Negative control				

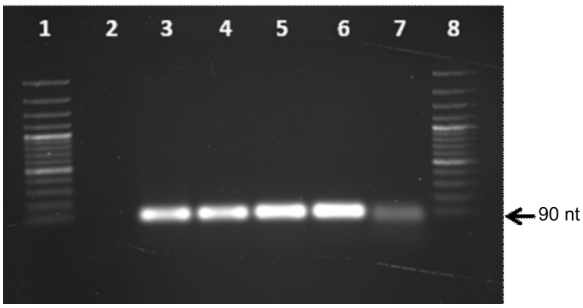

Figure 3 Agarose (0.7%) gel images for RT-PCR performed at virus dilutions Lane-1 and 8, DNA ladder (Fermentas, Lithuania); Lane-2, Blank; Lane-3, BHV-1 DNA 10^{-3} dilution; Lane-4, BHV-1 DNA 10^{-2} dilution; Lane-5, BHV-1 DNA 10^{-1} dilution; Lane-6, BHV-1 DNA pure dilution; Lane-7, negative control.

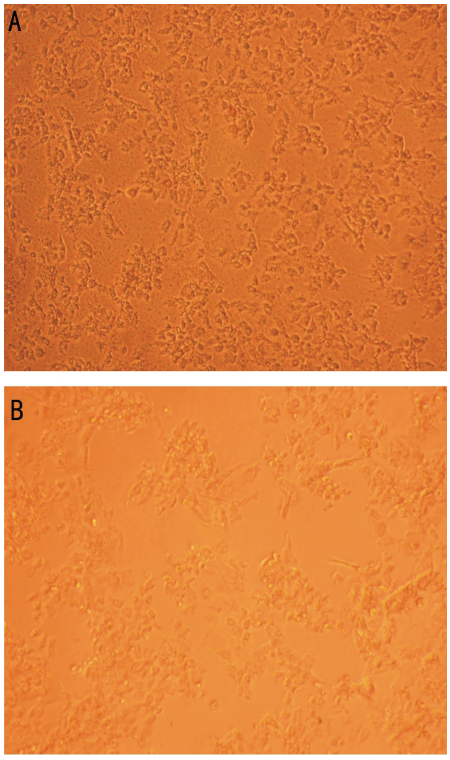

Figure 4 CPE (+) status A: V-A at 24h; B: CPE (+) status for V-A at 48h.

found to be negative for BHV-1 DNA. The V-HAM sample and positive control (PC) group had the same Ct values. Low Ct values indicate high DNA load as a result of high viral replication. However, more intense signal was observed for the V-HAM. Similar Ct values suggested that the amniotic membrane had no effect on virus replication. The V-A and

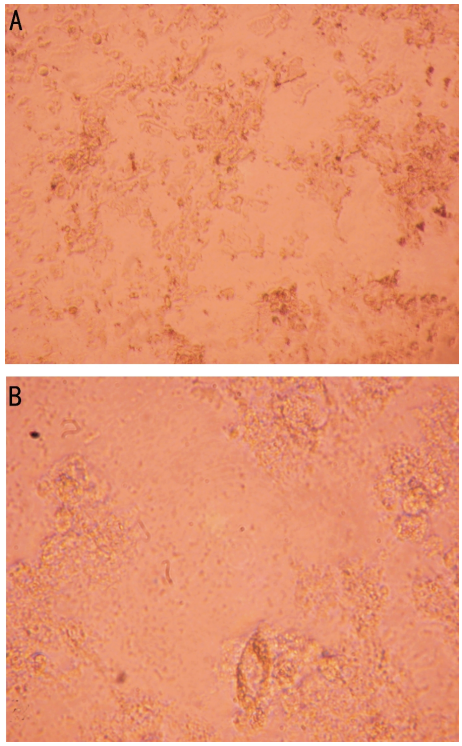

Figure 5 CPE (+) status A: V-HAM at 24h; B: 48h.

V-HAM-A samples had higher Ct values and less fluorescence than the PC samples due to the reduction in the load of DNA. The Ct of the V-A sample was slightly lower than that of the V-HAM-A sample, and it was concluded that the V-A treatment had no impact on the membrane. On the other hand, due to the barrier activity of the membrane in the V-HAM-A treatment, we expected that the viral DNA load was less for the V-A sample. Accordingly, the value of the Ct was predicted to be higher (Figure 4). However, it was difficult to assess the effect of the membrane in the V-HAM-A sample due to the late entry into the two cycles. We suggest that the number of experiments should be increased and the time points should be spread further apart.

To evaluate the effects of the amniotic membrane and acyclovir on cells, we included a control without virus cultivation in the second group of plates, which contained MDBK cells only;

A. Only HAM was applied to the petri dish. CPE (-) (C-HAM) (Figure 8).

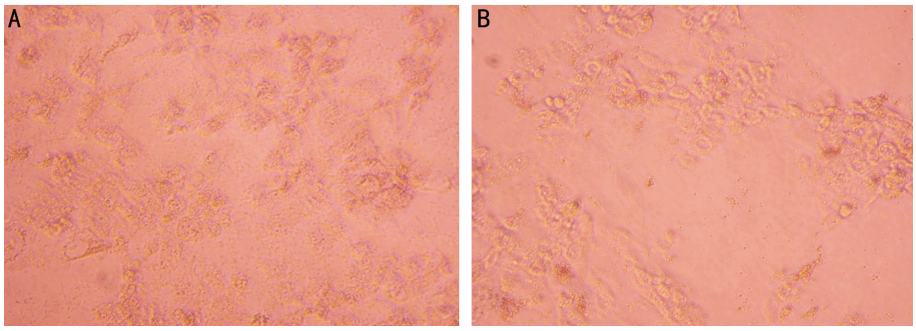

Figure 6 CPE (+) image A: V-HAM-A at 24h; B: 48h.

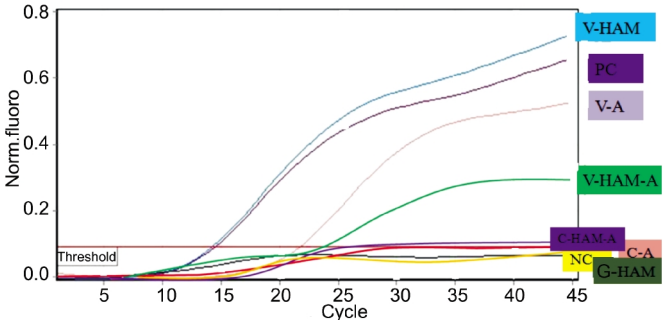

Figure 7 Amplification graph of RT−PCR analysis of groups 1 and 2.

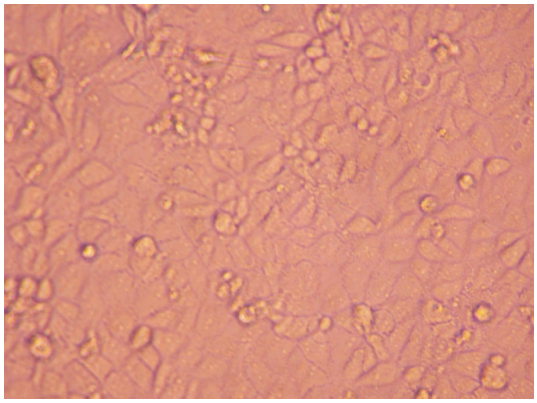

Figure 8 Microscopic images of CPE (−).

B. Acyclovir was applied to the HAM. CPE (-) (C-HAM-A) (Figure 8).

C. Only acyclovir was applied to the petri dish, CPE (-) was observed (C-A) (Figure 8).

There was no CPE (+) observed among the culture plates in the second group, and the application of the amniotic membrane and 3% acyclovir did not have a cytotoxic effect on the MDBK cells.

The fluorescence emission did not pass the threshold line, demonstrating that there was no viral load entering the replication cycle for the C-HAM, C-A and C-HAM-A samples (Figure 7).

DISCUSSION

To control the sight-threatening ocular infection caused by HSV, early diagnosis and effective treatment are very important [13]. Specific and rapid laboratory tests should be performed for the diagnosis of ocular herpes infection[14]. The newly developed RT-PCR techniques allow the detection of specific viruses with a fully automated and closed system, a low risk of contamination and a shorter time than PCR. RT-PCR can detect and quantify very small amounts of specific nucleic acid sequences [11,15-17]. RT-PCR is a useful method for diagnosing herpetic eye diseases based on quantification of the HSV DNA [4,18-21].

It has been reported that amniotic membrane has been successfully used in the treatment of corneal infections with herpes simplex virus [22-24]. Amniotic membrane and topical acyclovir therapy can be applied together for the cases showing a progressive improvement despite treatment and in complicated cases [8,9,24]. The amniotic basement membrane reduces the ocular surface inflammation and scarring, promotes rapid epithelialisation due to the presence of growth factors and has anti-inflammatory properties [25,26].

In our study, our aim was to investigate the impact of the membrane, the application of acyclovir to membrane and the use of acyclovir alone on the viral load. The amniotic membrane and acyclovir were prepared under sterile conditions in replicate experiments, and we observed that there was no toxic effect of the amniotic membrane or acyclovir on MDBK cells. RT-PCR analysis of V-HAM and the positive control (PC) group have the same Ct values and the viral DNA load was close to that of the PC, demonstrating that amniotic membrane had no antiviral effect.

In the study by Mencucci *et al* [27], the inhibitory effect of amniotic membranes incubated with the antiviral compounds on viral growth was investigated *in vitro*. It was observed that the amniotic membranes kept in a medium with an antiviral prevented viral growth in cell cultures infected with HSV. The membrane absorbed the antiviral and released drug [27].

In our study, the RT-PCR analysis following the application of acyclovir to the amniotic membrane showed that the viral DNA load was close to when the amniotic membrane was not used. Application of acyclovir to membrane indicated that membrane was permeable to acyclovir. It is difficult to confirm that the membrane had no barrier activity. More experiments are necessary with an extended time period.

In one study, AMT and antiviral treatments were applied to patients with necrotising ulceration due to HSV despite treatment with topical antiviral and lubricant therapy. The authors reported that all the stromal ulcers healed [8,9].

This study revealed that human amniotic membrane and acyclovir did not have a cytotoxic effect on cells. The results showed that the amniotic membrane alone had no antiviral effect. When the amniotic membrane was combined with the drug, membrane did not have a barrier effect to drug and was permeable to it.

In addition to medical treatment, factors secreted by a membrane or a basal membrane may have an effect on the clinical outcome of membrane lesions. By creating an animal keratitis model, RT-PCR analysis of the viral load can be performed in the cornea to examine the clinical improvement following the application of an amniotic membrane and drug. Thus, the effectiveness of the treatment can be evaluated and virus latency in the cornea can be controlled after clinical improvement. Similar applications should be tested in animal studies, and it can be recommended to evaluate the clinical effects of the amniotic membrane and acyclovir.

ACKNOWLEDGEMENTS

Conflicts of Interest: Kantarci FA, None; **Faraji AR,** None; **Ozkul A,** None; **Akata F,** None.

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