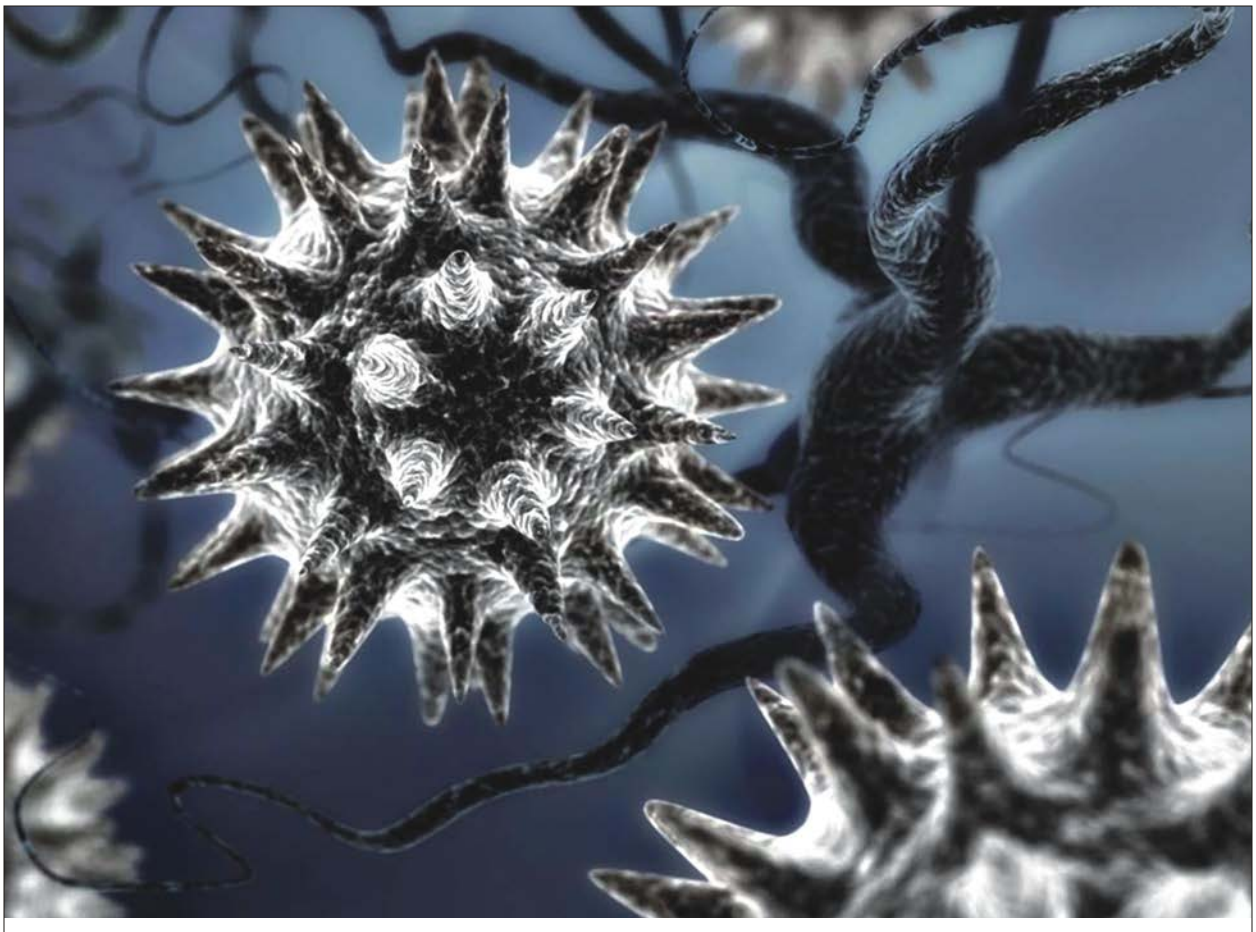

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Effect of Acyclovir on Bovine Herpesvirus Type 1 Infection in *in vitro* Cultured Cells

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ABSTRACT

Acyclovir (ACV) was screened for their inhibitory effect against Bovine Herpesvirus type 1 (BHV-1) *in vitro* in Madin-Darby Bovine Kidney (MDBK) cells using virus yield reduction test and recording cytopathic effect induced by BHV-1. The antiviral effect of ACV on BHV-1 was in dose dependant manner. Acyclovir at concentrations of 0.4, 0.5, 0.6 and 0.7 μg mL^{-1} reduced the Cytopathic Effect (CPE) of BHV-1 by 9, 17, 42 and 59%, respectively, in post infection treatment assay and by 17, 25, 50 and 64%, respectively in simultaneous infection treatment assay. While pre infection treatment assay showed that ACV had no effect on progression of CPE of BHV-1. ACV drastically decreased BHV-1 titers in dose dependent manner in both simultaneous and post infection treatment assays. In simultaneous treatment assay, there was significant decrease in virus titer by 0.35, 0.58, 1.44 and 2.34 logs due to concentrations 0.4,0.5,0.6,0.7 μg mL^{-1} of ACV, respectively. Also, ACV could decrease virus titer by 0.45, 1, 1.29 and 2.47 logs, respectively, in post infection treatment assay, while did not affect the virus titer in pre infection treatment assay. In conclusion, ACV was able to abolish virus titer and progression of CPE in MDBK cell cultures infected with BHV-1 when being added simultaneously and post viral infection.

Key words: Acyclovir, Madin-Darby bovine kidney cell culture, cytopathic effect, bovine herpesvirus type 1

INTRODUCTION

Bovine Herpesvirus-1 (BHV-1) is a member of the genus *Varicellovirus* in the subfamily Alphaherpesvirinae which belongs to the Herpesviridae family and order Herpesvirales. BHV-1 is known to cause several diseases worldwide in cattle, including rhinotracheitis, vaginitis, balanoposthitis, abortion, conjunctivitis and enteritis. BHV-1 is also a contributing factor in shipping fever, also known as bovine respiratory disease (Smits *et al*., 2000). This virus has worldwide economic impact on livestock industry (Jones, 2003). In Egypt, since 1960s, attention was drawn to BHV-1 as one of the most significant causes of great economic loss in feedlot and dairy farms, mainly due to death from pneumoenteritis, abortion, prolonged feeding periods because of weight loss, cost of treatment and prevention programs (Aly *et al*., 2003).

Acyclovir (9-{2-hydroxy (ethoxy) methyl} guanine) (ACV) is a preferable therapeutic drug used for treatment of several herpesvirus infections. It was developed in 1970s and becomes the agent of the first choice for treatment of herpes virus infections due to its significant efficacy and safety (De Jalo *et al*., 2003). It is a nucleoside analogue that exhibits anti-herpetic activity after

phosphorylation by viral thymidine kinase (Karpenko *et al*., 2003). ACV also plays an important role as broad spectrum antiviral agent which has virostatic activities against both DNA and RNA viruses, including herpesvirus, influenza virus, canine hepatitis virus (El-Gallad, 2008). Therefore there is a need to continue research to control BHV-1. The present work aimed to study the antiviral activity of ACV against BHV-1 infection *in vitro*.

MATERIALS AND METHODS

Antiviral agent: ACV was purchased as acyclovir 800 Stada (800 mg/capsule, Glubal Napi pharmaceuticals, Egypt). It was prepared as 10 mg mL^{-1} stock solution in deionized water. It was sterilized by filtration using Millipore filter (0.45 μm, Amicon) and used at different concentrations, including 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 μg mL^{-1}. All ACV solutions were stored at -20°C.

Virus and cell culture: Continuous cell line of MDBK cells were supplied by Egyptian Company for the production of serums and vaccines and medicines, Agouza, Giza and BHV-1 was kindly obtained from Virology Department, Faculty of Veterinary Medicine Zagazig University. MDBK cells were maintained at 37°C with 5% CO_2 for 24 h in culture flasks with MEM. Subcultured every 2-3 days after formation of confluent monolayer. BHV-1 was grown in MDBK cells which were also used for measurement of viral Infectivity by a dilution method using a 96-well microtiter plate. The infection titer was expressed as 50% Tissue Culture Infectious Dose ($TCID_{50}$) calculated by the formula of Reed and Muench (Reed and Muench, 1938). The infection titer of BHV-1 stock solution was 10^6 $TCID_{50}$ mL^{-1}.

Cytotoxicity assay: The toxicity of acyclovir was determined as following: confluent cultures of MDBK cells were grown in 96-well microtiter plate and exposed to different concentrations of ACV for 48 h at 37°C. Cell degeneration was evaluated during microscopic examination. Cytotoxicity was expressed as 50% Cytotoxic Concentration (CC_{50}); that is, the concentration of acyclovir required to reduce viral cytopathogenicity by 50% (Mucsi *et al*., 2001).

Assay of antiviral activity: The antiviral activity of acyclovir was investigated using MDBK cells. Confluent monolayer cultures of MDBK cells were grown in 24 well-plates and were infected with BHV-1 at a multiplicity of 10^6 $TCID_{50}$ mL^{-1} and treated with non cytotoxic dose of ACV acyclovir as described previously (Suzuki *et al*., 2006).

Preinfection treatment assay: Confluent monolayer cultures of MDBK cells were grown in 24 well-plates, treated with ACV, incubated for 24 h at 37°C and then infected with BHV-1. Results were examined after 3 days of inoculation. Percentages of Cytopathic Effect (CPE) were calculated as following:

$$\text{CPE (\%)} = \frac{\text{No. of wells having CPE}}{\text{Total No. of infected wells}} \times 100$$

Simultaneous infection treatment assay: Confluent monolayer cultures of MDBK cells were grown in 24 well-plates, treated with ACV and inoculated with BHV-1 simultaneously. Results were examined after 3 days of inoculation and %CPE was calculated as mentioned above.

Postinfection treatment assay: Confluent monolayer cultures of MDBK cells were grown in 24 well-plates, inoculated by BHV-1, incubated for 24 h at 37°C and then treated with ACV. The plates were incubated for 3 days and % CPE was calculated as mentioned above.

Virus yield reduction assay: Monolayer cultures of MDBK cells were grown in 24-well plates and were infected with BHV-1 at a multiplicity of infection of 0.5 $TCID_{50}$ per cell. After adsorption for 1 h at 37°C, the inoculum was removed and the cultures were washed twice with Phosphate Buffered Saline (PBS) and MEM containing different concentrations of ACV (three wells per concentration) was added. After incubation for 24 h the cultures were frozen at -70°C, then thawed and the cell debris was removed by low speed centrifugation. The virus yields in the supernatant were determined using MDBK cells by micro-well dilution method in 96-well plates. Control cultures were incubated with media without antiviral compound. The viral inhibitory effect was evaluated by comparing viral titers obtained in the presence of ACV at pre, simultaneous and post infections of BHV-1 (Mucsi *et al*., 2001).

RESULTS

Toxicity of ACV on cultured MDBK cells: Toxicity of ACV was preliminary tested against MDBK cells. ACV at concentrations of 0.1-0.7 μg mL^{-1} did not cause any change in the MDBK cells. However, higher concentrations of ACV (0.8-1.0 μg mL^{-1}) were found to cause MDBK cell rounding and clumping that could be considered toxic effect (Fig. 1a, b).

Effect of ACV on CPE of BHV-1: The effect of acyclovir at concentrations of 0.1-1.0 μg mL^{-1} on BHV-1 was tested using cultured MDBK cells and three infection protocols (pre, simultaneous and post infection treatment assays). Result are shown in Table 1 and 2. CPE values were 100% in pre infection treatment assay. However, CPE value reached 91, 83, 58, 41% in simultaneous assay and reached 83, 75, 50 and 36% in post treatment assay, respectively. This reflected an inhibition percentage of BHV-1 by 9, 17, 42 and 59% in simultaneous assay and by 17, 25, 50 and 64% in post treatment assay at concentrations of ACV of about 0.4,0.5,0.6 and 0.7 μg mL^{-1}, respectively.

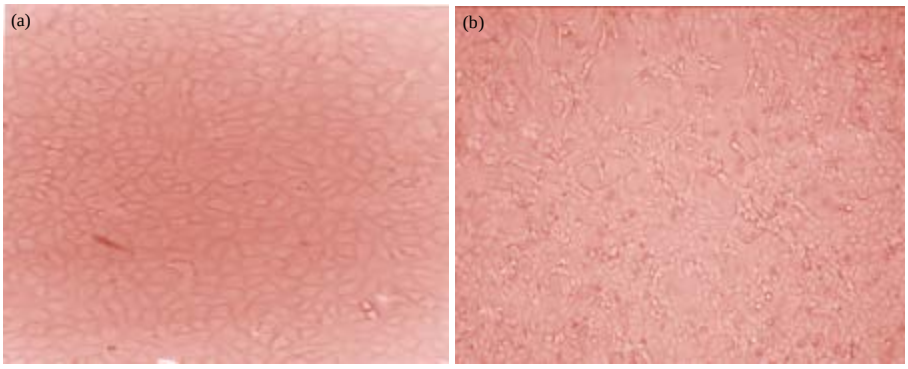

Fig. 1(a-b): Cytotoxicity effect of ACV on MDBK cells (X100), (a) Normal MDBK cell cultures showing confluent monolayer sheet of cells and (b) MDBK cells treated with high concentration of ACV showing cell degeneration

Table 1: The effect of ACV on induction of CPE in MDBK cells by BHV-1

	BHV-1 inoculation		
Conc. of ACV (μg mL^{-1})	Preinfection treatment assay	Simultaneous infection treatment assay	Postinfection treatment assay
0.1	CPE: 100%	CPE: 100%	CPE: 100%
0.2	CPE: 100%	CPE: 100%	CPE: 100%
0.3	CPE: 100%	CPE: 100%	CPE: 100%
0.4	CPE: 100%	CPE: 91%	CPE: 83%
0.5	CPE: 100%	CPE: 83%	CPE: 75%
0.6	CPE: 100%	CPE: 58%	CPE: 50%
0.7	CPE: 100%	CPE: 41%	CPE: 36%
Cell control	No CPE	No CPE	No CPE
Virus control	CPE	CPE	CPE

Table 2: Effect of ACV on BHV-1 titer*

	Inhibition of virus yield log10 $TCID_{50}$		
Concentration of ACV (μg mL^{-1})	Pretreatment assay (Before 24 h)	Simultaneous assay (at the same time)	Post treatment assay (after 24 h)
Control BHV-1	6.20	6.20	6.20
0.1	6.21	6.10	6.20
0.2	6.11	6.04	6.18
0.3	6.11	6.04	6.04
0.4	5.95	5.85	5.75
0.5	5.83	5.62	5.20
0.6	5.83	4.76	4.91
0.7	5.81	3.86	3.73

*ACV had a 1-3 log 10 inhibition of BHV-1 yield, depending on its concentrations

Effect of ACV on titer of BHV-1: In order to calculate the titer of BHV-1, different dilutions of ACV drug (0.1-0.7 μg mL^{-1}) and fixed virus dose of 10^6 $TCID_{50}$ mL^{-1} are used. The reduction of virus yield (log10 $TCID_{50}$) was determined. In pre infection treatment assay. It was observed that ACV has no effect on virus growth in comparison to infected non treated cells. In simultaneous and post infection treatment assays, there was significant decrease in virus titers as shown in Table 2.

DISCUSSION

BHV-1 is a worldwide contagious disease of economic and trade importance. In Egypt, first record of BHV-1 reported by Fatehya (1974). This virus has been reported in many provinces since then. Therefore there is a need to continue research to control BHV-1. This work was designed to study the effect of ACV drug as chemotherapeutic agent for controlling BHV-1 infection. ACV selectively inhibits herpes viruses that having a thymidine kinase The viral thymidine kinase activates the drug by phosphorylation into monophosphate and this is subsequently converted by cellular enzymes to be ACV triphosphate which is potent inhibitor of the viral DNA polymerase (Mucsi *et al*., 2001). Cytotoxicity of ACV was tested in MDBK monolayer cells and showed that ACV had cytotoxic effect on MDBK monolayer cells only at high concentration, while it had no cytotoxicity effect at 0.7 μg mL^{-1} or below as shown in the Fig. 1. If simultaneous application of antiviral compounds results in smaller doses being used, any cytotoxic effects of the drugs may also be reduced (Mucsi *et al*., 2001).

To assess whether ACV could inhibit BHV-1 replication, MDBK cells were treated with ACV at concentrations of 0.1-0.7 μg mL^{-1} at different periods of time. The results indicated that ACV was able to interfere with BHV-1 infection in simultaneous as well as post infection treatment assay. Acyclovir at concentrations of 0.4, 0.5, 0.6 and 0.7 μg mL^{-1} reduced the CPE of BHV-1 by 9, 17, 42 and 59%, respectively, in post infection treatment assay and also reduced the CPE of BHV-1 by 17, 25, 50 and 64% in simultaneous infection treatment assay, respectively, in comparison to pre infection treatment assay in which acyclovir had no effect on progression of CPE (Table 2). These results agreed with the finding of Mucsi *et al.* (2001) and indicated that ACV drug may affect BHV-1 replication. This observation was parallel to that recorded by Zheng *et al.* (2001) and Suzuki *et al.* (2006) who revealed that ACV is a substrate with high affinity for the viral thymidine kinase and a potent inhibitor of viral DNA synthesis.

In virus yield reduction assay, the virus titers were significantly decreased in MDBK cells in both simultaneous and post treatment infection assays. In simultaneous treatment assay, there was significant decrease of virus titers by 0.35, 0.58, 1.44 and 2.34 logs when ACV solutions at the concentrations of 0.4, 0.5, 0.6 and 0.7 μg mL^{-1} were added simultaneously with BHV-1 inoculation, respectively. Also it observed that the ACV had the ability to decrease virus titer by 0.45, 1, 1.29 and 2.47 logs, respectively, as shown in Table 2. These results agreed with the finding of Zheng *et al.* (2001). The positive antiviral control of acyclovir showed that the highest antiviral activity when added during the intracellular replication period, this drug inhibit specifically the viral DNA polymerase during the intracellular replication cycle when new viral DNA is synthesized (Nolkemper *et al.*, 2010). These data are in accordance with our results that demonstrated ACV when added simultaneous with virus infection and post viral infection, the virus titer was greatly reduced and abolish progression of CPE.

CONCLUSION

Present results demonstrated ACV when added simultaneous with virus infection and post viral infection, the virus titer was greatly reduced and abolish progression of CPE.

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