Does porphyrin suppres the apoptotic and necrotic effects of bovine herpes virus type-1(BoHV-1) and herpes simplex virus type-1(HSV-1)?

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Abstract: In this study**,** antiviral effect of porphyrin was investigated. Cooper strain of Bovine Herpes Virus type 1(BoHV-1) and Kos strain of Herpes Simplex Virus type-1 (HSV-1) were used to determine the potential of porphyrins to inhibit infection *in vitro* (with morphological and cytopathological criteria). Apoptotic and necrotic changes were determined by using DAPI and propidium staining. The non-cytotoxic dose of porphyrin (NCD-p) was initially calculated as 312.50µg/mL on MDBK and Vero cells. The apoptotic cell (APC) count was found 10% with BoHV-1 while it was 5.3% with BoHV-1 treated with porphyrin on MDBK cells between 6^{th} to 24^{th} hours post infection (hpi). Necrotic cell (NEC) count was 51% with BoHV-1 and 37.8% BoHV-1 treated with porphyrin on MDBK cells at 24^{th} hpi. On the other hand, the APC count was found 23% with HSV-1, while 22% with the HSV-1 treated with porphyrin on Vero cells between 6^{th} to 24^{th} hpi. NEC count was 49% with HSV-1 and 34% HSV-1 treated with porphyrin on MDBK cells at 24^{th} hpi. The results show that BoHV-1 was inhibited by porphyrin resulting in decreased apoptotic and necrotic changes in MDBK cells. On the contrary, porphyrine was not effective in the inhibition of HSV-1 in terms of apoptosis but it caused necrotic changes in Vero cells.

Keywords: Bovine Herpes Virus 1, Herpes Simplex Virus 1, porphyrin, apoptosis, necrosis.

INTRODUCTION

Bovine herpesvirus type 1 (BoHV-1) and Herpes simplex virus type 1 (HSV-1) are DNA viruses that belong to *alphaherpesvirinae* subfamily of the *Herpesviridae* family (Roizman 1996; Baxbaum *et al*., 2003; Maclachlan and Dubovi, 2011). BoHV-1, is a major pathogen of cattle associated with respiratory and genital tract infections such as infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), infectious balanoposthitis, and abortion (Tikoo *et al*., 1995; Ackermann and Engels, 2006; Nandi *et al*., 2009)**.** BoHV-1 may also cause central nervous system disorders which may lead to death of the newborn calves (Ackermann and Engels, 2006). The virus is able to establish latent infection in animals which become a major source of virus spread (Ackermann and Engels, 2006). HSV-1 is the human pathogen and causes a variety of clinical symptoms including gingivostomatitis, orolabial ulcers, vesicular eruptions of skin, conjunctivitis, keratitis, encephalitis etc (Igde *et al*., 2007). The virus infects epithelial cells and establishes a latent infection in neurons of trigeminal ganglia (Baskin *et al*., 2005).

Nucleoside analogues such as gangclovir, acyclovir, and derivatives are the most important antiviral drugs using the therapy of herpesvirus infections (Rybachuk, 2009). These compounds terminate viral DNA synthesis by inhibiting viral DNA polymerase (Baskin *et al*., 2005; Rybachuk, 2009).

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Porphyrins are a group of colored organic compounds which naturally occur and are used as photodynamically activated agents for the therapy of cancer, psoriasis macular degeneration, and cardio logical disorders (Lane, 2003; Rybachuk, 2009; Costa *et al*., 2012). These compounds show antiviral effects against some viruses, including retrovirus, influenza virus, hepadnavirus, etc. (Vzorov *et al*., 2002; Dixon *et al*., 2004; Lin and Hu., 2008; Wen *et al*., 2009). Antiviral mechanism of porphyrins is poorly understood. However, attachment of virus, entry into cells, fusion of cells in the virus replication cycle are inhibited by porphyrins (Vzorov *et al*., 2002; Dixon *et al*., 2004; Lin and Hu., 2008; Rybachuk 2009; Wen *et al*., 2009). Studies with HIV showed that the antiviral effects of porphyrins may be due to inhibition of the reverse transcriptase and HIV protease (Rybachuk, 2009; Vzorov *et al*., 2002). Furthermore, natural porphyrins may block HIV entry mediated by glycoprotein 120 interaction with $CD4^+$ (Vzorov *et al*., 2002).

The aim of this study was to investigate the potential of porphyrins to inhibit infection by BoHV-1 and HSV-1 in cell culture (with morphological and cytopathological criteria).

MATERIALS AND METHODS

Porphyrin: 5, 10, 15, 20 Tetrakis (4-trimethylammonio-phenyl)-21*H*, 23*H*-porphine (TTMAPP) were purchased from Sigma-Aldrich (USA). TTMAPP was dissolved in dimethyl sulfoxide (DMSO) and prepared at a concentration of 5000µg/mL. Stock solution was protected from light until further use.

Cell Line and propagation of viruses

MDBK (Madin Darby Bovine Kidney) and Vero (African Green Monkey Kidney) cell lines were maintained in DMEM (Dulbecco's Modified Eagle's Medium, Biological Industries, Israel) supplemented with 10% FBS (Fetal Bovine Serum, Biological Industries, Israel) and 1% antibiotics (Biological Industries, Israel) incubated at 37°C in 95% humidity and 5% CO_2. MDBK was used for the for the propagation of BoHV-1 (Cooper strain) and Vero was used for HSV-1(KOS strain). Both cell cultures containing viruses were incubated in DMEM with 2% FBS at 37°C and examined daily with an inverted microscope (Krüss, Germany) until the cytopathic effects reached 80-100%. After freezing and thawing thrice, virus-cell lysates were distributed into aliquots and stored at -80°C as virus stocks (Yazici *et al*., 2004; Baskin *et al*., 2005).

Determination of infectivity of viruses

Infectivity of BoHV-1 and HSV-1 was determined by using the micro titration technique on MDBK and Vero cells in different 96 well tissue culture plates for each virus (TPP, Switzerland) as described by Frey and Liess (1971). Briefly, tenfold dilution was prepared for each virus. 100 µL of each dilution were put into in quadruplicate in 96 wells. Than 50µL of cell suspensions were added to each well and the plates were incubated at 37°C for 72 hours. After incubation, 50% tissue culture infective dose were calculated by the method of Reed and Muench (1938) and expressed as $Log_{10}TCID_{50}$/ml.

Determination of Non Cytotoxic dose of porphyrin (NCD-p)

The NCD-p (Non Cytotoxic dose of porphyrin*)* was determined for MDBK and Vero cells by using a micro titration plate technique (Yazici *et al*., 2004). In separate plates, two fold dilutions of porhyrin with DMEM were prepared from 5000µg/mL to 1.25ng/mL. Equal amounts of each dilutions were put into in quadruplicate in 96 wells. Subsequently, 100µL of MDBK cell suspensions (450,000cells/mL) were added to each dilution. The same procedure was performed for the Vero cells (600,000cells/mL). The plates were then incubated at 37°C for 48 hours. The cells were examined daily with inverted microscope. The NCD-p was determined by staining the cells with the vital dye trypan blue. At toxic doses, blue color was not observed in the cells because of cell death, but at non-toxic doses the cells were colored blue. These experiments were repeated three times.

Treatmen of BoHV-1 and HSV-1 with Porphyrine

NCD-p and $TCID_{50}$ doses of BoHV-1 and HSV-1 were equally mixed and were incubated in dark at room temperature (RT) for 1h. MDBK and Vero cells were grown in separate 24 well tissue culture plates (Nunclon, Denmark). Following cell confluence, NCD-p/BoHV-1 combination was inoculated on MDBK cells and incubated at 0.5, 1, 3, 6 and 24 hpi. The same procedure was performed for NCD-p/HSV-1 on the Vero cells. For the morphological assesment of antiviral effect of porhyrine, apoptotic and necrotic cells were investigated using vital dyes DAPI (4',6- Diamidino-2- phenylindole dihydrochloride) (Sigma-Aldrich, USA)**,** and propidium iodide (Sigma-Aldrich, USA). Briefly, for the detection of apototic cells, the cells were fixed with cold ethanol at RT for 10 min. The ethanol was aspirated and cells were washed thrice with PBS and stained with 0.5µg/mL DAPI for 5 min at 37°C. For the detection of necrotic cells; the cells were fixed with cold ethanol at RT for 10 min. The ethanol was aspirated and cells were washed three times with PBS and stained with 1.0µg/mL propidium iodide (PI) for 30 min at 37°C. At the end of DAPI and PI staining, the cells were monitored using a fluorescent microscope at the wavelength of 330 and 420 nm. This experiment was repeated three times and at least 200 cells were counted in five different areas of each well (Baskin *et al*., 2005)l The cells were classified into three types according to the criteria as follows (Baskin *et al*., 2005):

Live cells: Normal nuclei, pale blue/green chromatin with an organized structure. *Apoptotic cells:* Chromatin condensation within the nucleus, intact nuclear boundaries, bright blue chromatin and nuclear fragmentation into smaller nuclear bodies within an intact cytoplasmic membrane.

Necrotic cells: Damage to the cytoplasmic membrane with a fairly intact nucleus, enlarged red nuclei, loss of the cytoplasm, damaged/ irregular nuclear membranes and slightly condensed nuclei that are stained in bright red color.

RESULTS

Infectivity of BoHV-1 and HSV-1

BoHV-1 and HSV- 1 infectivity were determined as $TCID_{50}$ $10^{-4.50}$/0.1mL and $10^{-3,5}$/0.1mL using micro titration method, respectively.

Determination of non-cytotoxic doses of porphyrine (NCD-p)

NCD-p was determined as 0.31250mg/ml in both cell cultures.

Effect of NCD-p for cell cultures

APC, NEC, and LC counts were determined as 8-15%, 10-24% and 61-82%, respectively after NCD-p (0,31250 mg/mL) inoculated on MDBK cells between 0.5 to 24 hpi. These rates were lower than the control cells of MDBK (w/o porphyrine) calculating 9-19%, 4-12% and 69-87%, respectively. APC, NEC, and LC counts were determined as 8,5-14%, 12-16%, and 70-79%, respectively after NCD-p (0,31250 mg/ml) inoculated on Vero cells between 0.5 to 24 hpi. These rates were lower

than the control cells of Vero (w/o porphyrin) calculating 7-12.3%, 6-11, %, and 76-87%, respectively.

Determination of effect of for BoHV-1 and HSV-1 in Cell Cultures

APC, NEC, and LC counts were determined as 8,5-29%, 1,5-51%, and 20-90% respectively, between 0.5 to 24 hpi on MDBK cells infected with $TCID_{50}$ of BoHV-1 (fig. 1), while 9-23%, 2,9-49% and 1-88%, respectively for $TCID_{50}$ of HSV-1 at the same period (fig. 2). According to obtained results, apoptosis and necrosis were induced with BoHV-1 and HSV-1 in MDBK and Vero cells at the 6 hpi and LC count was descreased at the same time period.

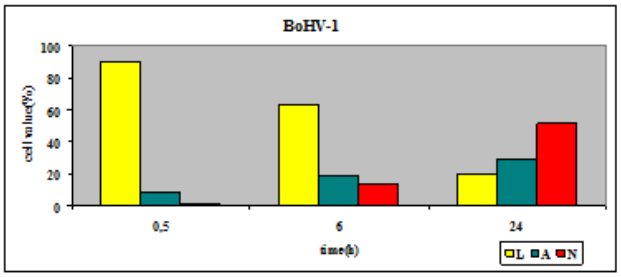

Fig. 1: Cell count (%) between 30 min to 24h post-infection on MDBK cells after inoculation with $TCID_{50}$ of BoHV-1. A: Apoptotic Cells, N: Necrotic Cells, L: Live cells.

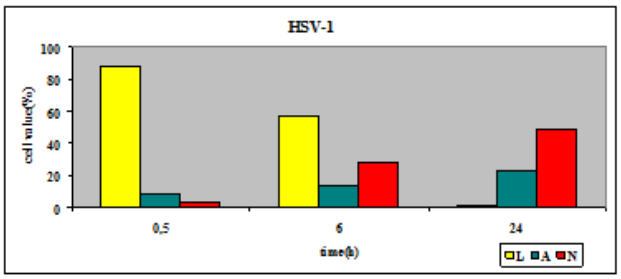

Fig. 2: Cell count (%) between 30 min to 24h post-infection on vero cells after inoculation with $TCID_{50}$ of HSV-1 A: Apoptotic Cells, N: Necrotic Cells, L: Live cells.

Determination of the antiviral effect of porphyrin for BoHV-1 and HSV-1 in cell cultures

APC, NEC, and LC counts were determined as 7,4-23,3%, 0-37,8%, and 39-89% between 0.5 to 24 hpi on MDBK cells infected NCD-p /BoHV-1 (fig. 3). These rates were found as 6,2-22%, 2,2-34% and 44-91% on Vero cells infected NCD-p HSV-1(fig. 4). By the comparison, apoptosis and necrosis were also induced with NCD-p/BoHV-1 and NCD-p/HSV-1 at the 6 hpi on MDBK and Vero cells, but obtained counts were lower than both viruses untreated NCD-p.

DISCUSSION

At the present time, porphyrins are being used for treatment of cancer and dermatological disease. Particularly, these compounds are described as tumor localized photosensitizer molecules which react with molecular oxygen and other substrates to generate highly cytotoxic species that interact with cellular components and ultimately, result in cell death and destruction of tumor tissue (Rybachuk, 2009). On the other hand, porphyrins have antiviral activity on viruses including retroviruses, adenoviruses and paramyxoviruses (Rybachuk, 2009; Dixon *et al*., 2004; Vodzinska *et al*., 2011). The antiviral property of porphyrins may be due to inhibition of viral replication and interaction of cell receptors with viral enzymes (Rybachuk, 2009). Previously, Vzorov *et al* (2002) investigated the anti-HIV effect of porphyrins. They reported that the binding of envelope proteins to cells and fusion capabilities were inhibited by porphyrins. In another study, researchers showed that supression of replication of HSV-1 and 2 is increased 40% by glycoporphyrins (Tome *et al*., 2005). Antiviral effect of *meso*-tetraphenyporphyrin-2 and *meso*-tetraphenyporphyrin-3 on HSV-1 is reported by Silva *et al* (2005). Porphyrin compouns also inhibited the 70% replication of EHV-1 (Rybachuk, 2009). According to Lin and Hu (2008), activity of reverse transcriptase in replication cycles of *hepadnavirus was* descreased by 50%.

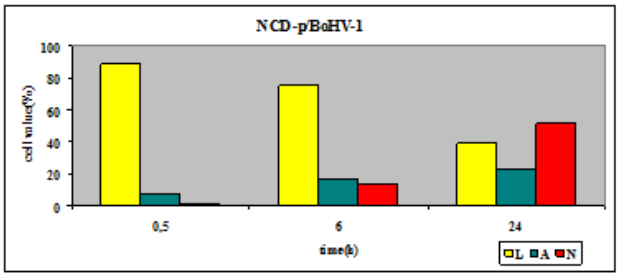

Fig. 3: Cell count (%) between 30 min to 24h postinfection on MDBK cells after inoculation with BoHV-1 treated with NCD-p A: Apoptotic Cells, N: Necrotic Cells, L: Live cells.

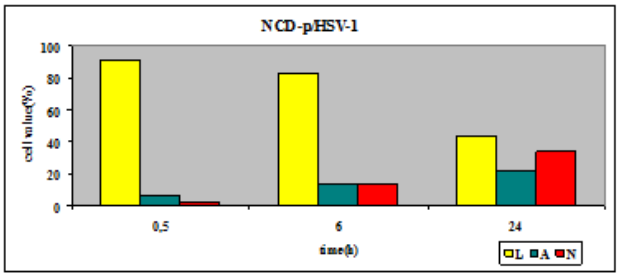

Fig. 4: Cell count (%) between 30 min to 24h post-infection in Vero cells after inoculation of HSV-1 treated with NCD-p. A: Apoptotic Cells, N: Necrotic Cells, L: Live cells.

In the present study, NCD -p was found to have the same value in the both cell cultures. APC count was between 8% to 15% for MDBK cells and 8.5% to 14% for Vero cells while NEC count was 10% to 24% and 12% to 16

respectively. Obtained APC values were close in control group of MDBK and Vero cell line determined by physological apoptotic cell value. On the contrary, the NEC value of control cell cultures was found less than cell cultures treated.

In the current study, APCs were determined as 8.5% at 0.5 hpi, 19% at 6 hpi, 29% at 24 hpi while NECs were as 1.5%, 14% and 51% at the same periods in MDBK cells with BoHV-1. Between 0.5h to 6 hpi, an increase of APC count was balanced but it was lower than 6 hpi to 24 hpi. At the same time intervals, NECs was higher than APCs. This data showed that APCs and NECs were increased on BoHV-1 infection at first 6 hpi and NECs exceeded than APCs with the increase in post-infection time. An increase in APCs and NECs were observed in the Vero cells infected with HSV-1 at 6 hpi, but NEC count was more significant than APC count. At the same time, APC count was detected by 9% at 0.5 hpi, 17% at 6 hpi, and at 23% at 24 hpi, respectively. NEC count was 2.9% at 0.5 hpi, 28% at 6 hpi and 49% at 24 hpi.

APC value was 7.4%, 16.1%, 23.3% at 0.5 hpi, 6 hpi and 24 hpi with NCD-p /BoHV-1in MDBK, respectively. APC value was increased to 11.5% between 0.5 hpi to 6 hpi and 10% between 6 hpi to 24 hpi with BoHV-1 while these values were 8.7% and 7.2% with NCD-p /BoHV-1 in the same period. NEC values were found 9.6%, 37.8% at 6 hpi and 24 hpi with NCD-p /BoHV-1while NEC was not observed at 0.5 hpi. NEC value with NCD-p/BoHV-1 was lower than BoHV-1. The results indicated that the apoptotic and necrotic effects of BoHV-1 were suppressed by porphyrin. However, necrotic changes were more significantly inhibited than apoptosis in MDBK cells by porhphrins.

APC value was found 6.2%, 14%, 22% while NEC value was found 2.2%, 13%, 34% with NCD-p/ HSV-1 at the same time period. Although the effect of porphyrin was not clear, APC value with NCD-p/ HSV-1 was lower than HSV-1 in Vero cells. At the last observation time, NEC value was decreased by %15 by NCD-p/ HSV-1.

The results obtained from our study were similar to two different viruses of the same subfamily in *herpesviridae* family. We observed that porphyrins were able to supress the apoptotic and necrotic changes induced by both viruses. However, supression of apoptosis in BoHV-1 infection was more than HSV-1. Necrotic changes for both viruses were found close in value and these changes were increased at 6 hpi. This data showed that necrotic changes may play important role in the pathogenesis of BoHV-1 and HSV-1 *in vitro*. (Yazici *et al* 2004; Baskin *et al*., 2005) These results are also consistent with previous study by Baskin *et al* (2005).

CONCLUSION

The several compounds including porphyrins are being tried to develop treatment of viral infections. Currently, porphyrins and its compounds are in use for treating cancer and various diseases. This study showed that porphyrins may suppress apoptotic and necrotic effects of viruses. In the future, porphyrins can be represent attractive candidates as antiviral agents to treatment of viral infections.

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