Antiviral Competence of Broad-Spectrum Antiinfective Drug Artesunate against Bovine Herpesvirus-1

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Abstract: An *in-vitro* and *in-vivo* evaluation of the antiviral activity of Artesunate (ART) as a natural antiviral agent against Bovine Herpes Virus-1 (BoHV-1) and compared its activity in-vitro against Ribavirin. *In-vitro* evaluation was done on the MDBK cell culture system. Artesunate was able to reduce the release of the virus which was obvious in dropping of the titer of the released virus from cell culture system. Regarding Ribavirin, it showed an antiviral activity on the BoHV-1 but with lower response than that of Artesunate. *In-vivo* evaluation, three groups of rabbits were used as a laboratory animal's model. The clinical signs of the rabbits and histopathological examination were done on lungs collected from rabbits which were treated with Artesunate, showed a noticeable antiviral activity response against BoHV-1, while those infected with BoHV-1 without Artesunate treatment showed pneumonia and bad general health condition. However, molecular detection by the gel-based PCR was able to detect BoHV-1 in the lung tissues of both groups.

Key words: Bovine respiratory disease (BRD) · Cattle mortality · Bronchopneumonia · Infectious bovine rhinotracheitis · Infectious pustular vulvovaginitis

INTRODUCTION

Bovine respiratory disease (BRD) complex is a major health problem of cattle worldwide [1,2]. It inflicts considerable financial losses in beef herds and is the most common cause of mortality in dairy cattle [3]. The causality is multifactorial and the disease appears to be a result of the interaction of infectious micro-organisms and such predisposing factors as host defense, environment and stress [3]. Bovine herpes virus type 1 (BoHV-1) has been considered to be an important BRD-associated pathogens [4,5]. Apart from respiratory disease, this virus can cause other clinical syndromes such as infectious pustular vulvovaginitis or balanoposthitis, conjunctivitis, encephalitis and generalized systemic infections [6]. In adult cows, infection is associated with a severe and prolonged decrease in milk yield, reduced fertility and abortions while many cases run with a subclinical course [7].

BoHV-1 induces immunosuppression frequently leads to secondary bacterial infections (*Pasteurella haemolytica*, *Pasteurella multocida* and *Haemophilus somnus*) that can cause pneumonia [8]. Experimental evidence of field observations supported the involvement of BoHV-1 infection in promoting bacterial super infections leading to severe bronchopneumonia [9] and after primary infection with BoHV-1, cattle become latent carriers. BoHV-1 establishes lifelong latency in sensory neurons of the peripheral nervous system after replication in mucosal epithelium [10].

Due to the economical level of the developing countries, vaccines against BRD complex are used infrequently and sick animals mostly treated individually with antibiotics [11]. When reviewing the possibilities for usage of antiviral chemotherapy, the genetic material of the virus uses the biochemical mechanisms of the host cell to replicate its own genetic material. This replication step differs in RNA and DNA viruses, but mRNA is

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produced in all. Although all viruses follow this replicative cycle, different virus families may differ considerably from one to another at one or more steps of the cycle. Useful inhibitors are generally specific for one family of virus. The target of these antiviral agents can be: (1) Specific viral proteins, (2) Host cellular enzymes or (3) Modulation of host immune responses. Many scientists had dealt with antiviral agents with different mode of actions; one of these agents is Ribavirin (RBV), it is a well-known broad spectrum antiviral agent. Murphy *et al.*[12] , Durantel *et al.* [13] and Woodhouse *et al.* [14] have used Ribavirin (RBV) alone and in combination with other antiviral agents to eradicate many viruses and obtained controversial results.

Traditional Chinese medicine commands a unique position among all traditional medicines because of its 5000 years of history. Artemisinin-type sesquiterpene lactones from Artemisia annua, as demonstrated in recent years, this class of compounds has activity against malaria, cancer cells and schistosomiasis. Interestingly, the bioactivity of Artemisinin and its semisynthetic derivatives are even broader and include the inhibition of certain viruses [15].

Because Artemisinin itself has physical properties such as poor bioavailability that limit its effectiveness, semisynthetic derivatives of Artemisinin have been developed. These derivatives include Artesunate which is a water soluble salt and can be given by the oral, intramuscular, intravenous and even intra-rectal routes [16-18]. Artesunate inhibits the in-vitro replication of Human Cytomegalovirus (HCMV) and herpes simplex virus type 1 (HSV-1) [19]. Novel data show that other herpes viruses from all subfamilies (a, b and g) are also sensitive to Artesunate namely, Epstein-Barr virus, herpes simplex virus 1 and human herpes virus 6A [19,20]. These findings suggest that Artesunate has broad activity against herpes viruses than its natural parental drug, Artemisinin.

Therefore, this study aimed to run an in-vitro and in-vivo evaluation of the antiviral activity of the Artesunate on the BoHV-1. Besides, well-known broad spectrum antiviral RBV, as a model to compare the Artesunate activity against it.

MATERIALS AND METHODS

Materials
Antiviral Agents: Two antiviral agents were used in this study. One agent is a commercial product extracted from Artemesia annua, Aretesunate (Sigma-Aldrich Chemie GmbH). The other antiviral agent is a synthetic product, Ribavirin (MP Biomedicals, LLC). France.

Cell Culture System: Cell culture system which was used in this study to evaluate the in-vitro activity of both antiviral agents was the Madin Darby Bovine Kidney (MDBK) cell line which obtained from " Veterinary Sera and Vaccines Research Institute (VSVRI), Cairo, Egypt". The cells were maintained on the Growth media, 10% of heat-inactivated Fetal Bovine Serum (FBS) EU Standard-Lonza Bioproducts, Belgium and Pen /Strep Amphotericin B100 x, Lonza Bioproducts, Belgium. Cells were grown at 37°C in a humidified atmosphere of 10% CO2.

Virus: Bovine herpes virus type 1 (BoHV-1), Abou Hammad strain ($10^{7.5}$ $TCID_{50}$/ml). The virus used in the study was obtained from VSVRI.

Experimental Animals: Three groups (A,B and C) of male New Zealand white rabbits, 5 rabbits each, weighing about 3 kilograms, six months age were used. Animals were monitored clinically during the acute infection. Rabbits were kept until the onset of acute infection symptoms.

Methods
Preparation of Antiviral Agents: Stock solutions from Artesunate were prepared in 100% dimethyl sulfoxide (DMSO) HPLC Lab-scan. Artesunate was prepared at stock solution 65.033 mM then three concentrations were prepared as follow 1.5, 10, 20μM according to Bachmeier *et al*. [21].

Ribavirin was prepared in aqueous solution. It was prepared at stock solution of 10.2375mM then three concentrations were prepared as follow 50, 200, 400mM according to Durantel *et al*.[13]. Both stock and dilutions were sterilized by filtering through Syringe filters 0.2μm and stored at -20°C according to Milbradt *et al*. [22].

Plaque Forming Assay [23]: The assay was used to titrate both the reference virus, BoHV-1 and the virus outcome from in-vitro and in-vivo experiments. The BoHV-1 virus was serially diluted in PBS into 10-fold dilution.

DNA Extraction: The extraction was performed as indicated by the instructions in the QIAamp DNA minikit (Qiagen). BoHV-1 was extracted from rabbit lung tissues.

Gel-Based PCR [24]: The assay was conducted on the extracts from lung tissues to detect BoHV-1 as the main cause of rabbit lung pneumonia. The 468 bp fragment of the BoHV-1 gI glycoprotein gene was selected for amplification. Forward and Reverse oligonucleotides were designed and reproduced in a HPLC quality. The oligos are as follow,

F 5'- GCACAAGGCGAAAAAGAGCA-3' and R 5'-AGGAAAAACAAACAGCCGCA-3'. The PCR was carried out according to Emerald Amp GT PCR Master Mix. Each primer was added in 0.2µM, specified concentration from DNA template and the reaction completed up to 25µl by the dH2O. The reaction was as following, 1 cycle of 95°C for 2 min, 35 cycles of 95°C for 50 sec, 56°C for 50 sec, 72°C for 70 sec and final extension step at 72°C for 10 min. Then the PCR product was kept in 4°C till run the electrophoresis. The amplified product was visualized in 2% agarose gel then stained by Ethidium bromide to detect the specified band.

Histopathological Examination *In-vivo* Study: Rabbits were randomly allocated into three equal groups (n=5). Group A was kept as a negative control group and injected with diluted DMSO in sterile saline . Group B was a positive control group which is infected intranasal and by eye droppings with virus. Group C was infected with the BoHV-1 virus intranasal and by eye droppings then after two days this group was injected with Artesunate 2.0 mg/kg for five days [15]. Lungs from each

group were collected after a week and fixed in 10% neutral buffered formalin for preparing paraffin tissue sections at 4-6µ thickness. These sections were stained with hematoxylin and eosin. [25]. Rabbit lung tissues were sampled for detection of BoHV-1 by PCR as a cause of pneumonia [26].

RESULTS

Cytotoxicity Effect of Antiviral Agents: Artesunate and Ribavirin were applied with different concentrations on the cell culture system without inoculation of the virus to evaluate the toxicity effect of the agents. Both agents with different concentrations showed no toxicity on the cells for the whole 72 hours (Table 1).

Antiviral Activity Against BoHV-1: MDBK cell culture plates (four wells for each dilution) were cultivated in MEM medium containing FBS, Pen /Strep and Amphotericin. One day prior to infection, cells were treated with Artesunate, Ribavirin or equivalent amounts of DMSO. Inoculation with BoHV-1 was inoculated on the MDBK cells and incubated for 90 min at 37°C for virus adsorption. Thereafter, virus inoculum was not removed, but replenished with fresh medium containing the drugs with different concentrations. Plaque Forming Assay was used For virus titration as previously mentioned. Results of virus inhibition are recorded in (Table 2).

Table 1: Cytotoxicity of Artesunate and Ribavirin on MDBK tissue culture cells

Microscopic Changes					
Drugs	Concentrations	12hr	24hr	48hr	72hr
Artesunate	1.5 µM	-ve CPE	-ve CPE	-ve CPE	-ve CPE
	10 µM	-ve CPE	-ve CPE	-ve CPE	-ve CPE
	20 µM	-ve CPE	-ve CPE	-ve CPE	-ve CPE
Ribavirin	50 mM	-ve CPE	-ve CPE	-ve CPE	-ve CPE
	200 mM	-ve CPE	-ve CPE	-ve CPE	-ve CPE
	400 mM	-ve CPE	-ve CPE	-ve CPE	-ve CPE

Table 2: Antiviral activity against BoHV-1 in PFU/ml

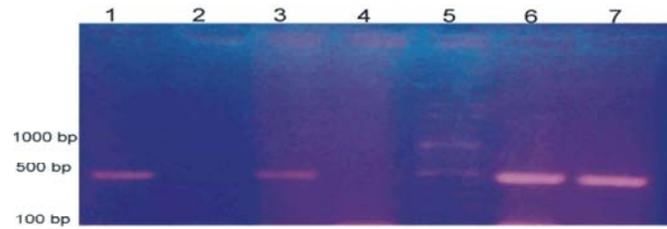| BoHV-1 titer by PFU/ml					
Drugs	Concentrations	12hr	24hr	48hr	72hr
Artesunate	1.5 µM	$7X10^6$	$4X10^5$	$4X10^5$	$3X10^4$
	10 µM	$6X10^6$	$6X10^5$	$4X10^5$	$3X10^3$
	20 µM	$5X10^6$	$2X10^6$	$2X10^3$	Negative
Ribavirin	50 mM	$7X10^6$	$7X10^6$	$7X10^5$	$6X10^5$
	200 mM	$7X10^6$	$7X10^5$	$4X10^5$	$3X10^4$
	400 mM	$5X10^6$	$5X10^6$	$4X10^5$	Negative

Fig. 1: Amplified PCR product of the 468 bp BoHV-1 gI glycoprotein gene fragment. Lane 5, 100bp ladder. Lane 1, control positive BoHV-1. Lanes,3, 6 BoHV-1 in infected rabbit lung. Lane 7 , BoHV-1 detected in lung of artisunate treated while Lane 2 and 4 are BoHV-1 negative

Fig. 2: Lung of control (injected with diluted DMSO in sterile saline), Artesunate (rabbit inoculated with both Artesunate and BoHV-1) and Infected (control positive rabbit)
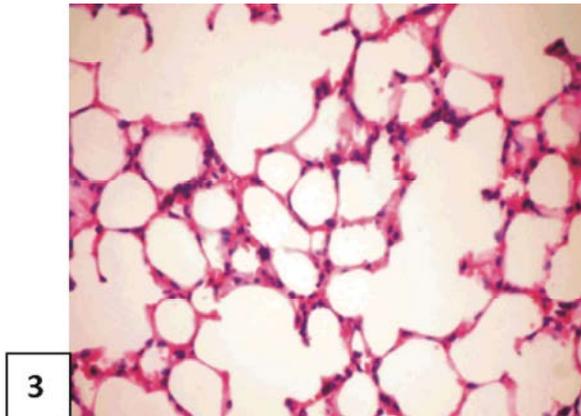

Fig. 3: Lung of negative control rabbits showing healthy pulmonary tissue (H&E X 400)
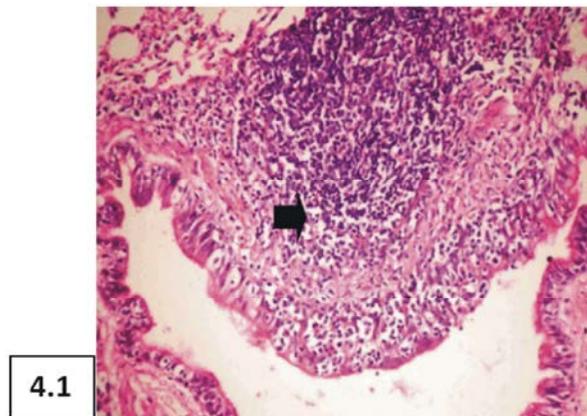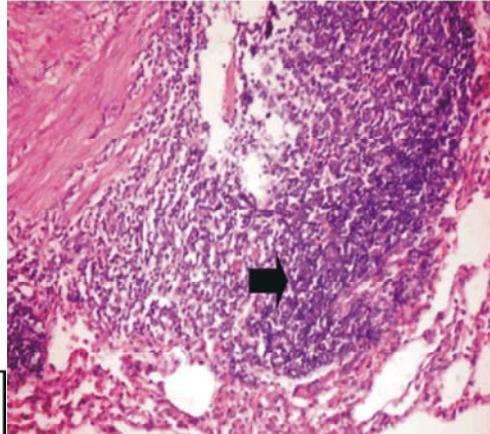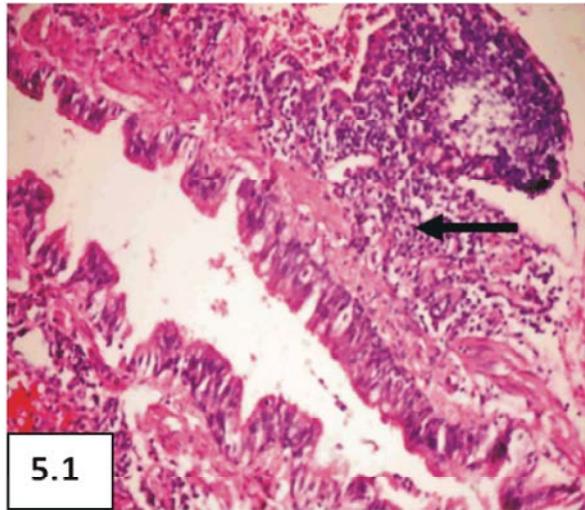

Fig. 4.1: Lung of infected rabbits showing severe viral pneumonia which represented by hyperplasia of the peri-bronchial lymphoid tissue (arrow head), (H&E X 400)
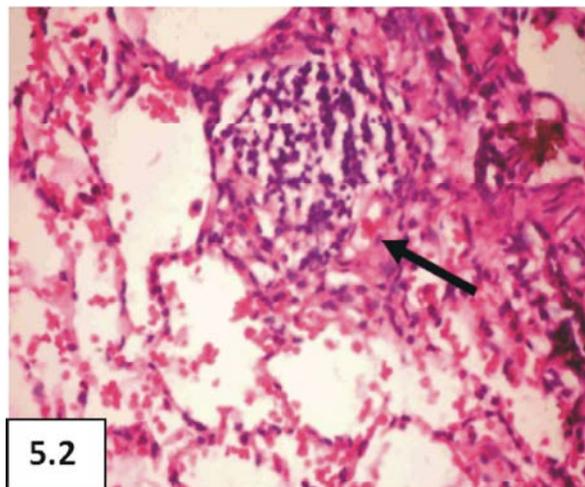
Fig. 4.2: Lung of infected rabbits showing diffuse lymphocytic infiltration in the interstitial tissues (arrow head), (H&E X 400)

Fig. 5.1: Lung of virus infected rabbits treated with Artesunate showing regression in the peri-bronchial hyperplased lymphoid tissue (arrow),(H&E X 400)

Fig. 5.2: Lung of virus infected rabbits treated with Artesunate showing regression in the interstitial infiltrated lymphocytes (arrow),(H&E X 400)

Detection of the Virus Yield by PCR: Figure 1 shows the specific band of the gI glycoprotein of the BoHV-1.

Histopathological Changes of In-vivo Activity of Artesunate

Post Mortem Lesions: Lung of viral infected group was edematous and congested than that of control negative group. While lung from Artesunate treated group was apparently similar to that of the control negative group Figure 2.

Histopathological Examination: Regarding lungs of rabbits from control negative group which injected with diluted DMSO in sterile saline, There were no histopathological alterations recorded (Figure 3). While Lungs of rabbits from virus infected group showing severe viral pneumonia which represented by hyperplasia of the peribronchial lymphoid tissue and diffuse lymphocytic infiltration in the interstitial tissues (Figure 4.1 & 4.2). After Artisunate treatment, lungs microscopy revealed regression in the peribronchial hyperplased lymphoid tissue together with regression in the interstitial infiltrated lymphocytes (Figure 5.1 & 5.2).

DISCUSSION

This study revealed that there is a pronounced antiviral activity of Artesunate against BoHV-1 which should be added to the previously published broad anti-herpes viral activity. The aim of this study is to evaluate the antiviral activity of the Artesunate in-vitro and in-vivo on the BoHV-1.

Toxic effect of Artesunate or Ribavirin on the cells and lab animal, rabbits were examined . This done to eliminate cytotoxicity effect before running the experiments. Results indicated both agents with above mentioned concentrations had no toxicity neither on the cells culture system for 72 hours nor the rabbits. But, when we attempted to subculture the treated cells, it failed to grow again. This may indicate that we have to reduce the concentration less than 10μM in Artesunate and 100mM in Ribavirin. This may be referring to the effect of the agents on the genomic materials of the cells. Our results are running in the same outcomes that gained by Murphy et al. [27] and Sarciron et al. [28]. They proved that both agents did not produce any cytotoxicity in their cells.

Based on CPE inhibition of MDBK cells, the antiviral activity against BoHV-1 was evaluated in three different ways according to the time and orders of applications of both antiviral agents and virus. Results indicate that Artesunate has a potential antiviral activity against BoHV-1 with different concentrations; these results are in the same manner with that Ribavirin except the Artesunate showed more marked effect on the yield of the virus from MDBK cells. These results are in accord with that obtained from Milbradt et al. [22] and Efferth et al. [19]. They stated that the cells infected with herpes virus in the study and treated with different concentrations of Artesunate, showed pronounced inhibitory effect on the virus yield.

The antiviral activity of Artesunate was also demonstrated in the rabbit as an animal model which further confirmed the lack of toxicity of Artesunate treatment. Severe pneumonia was recognized in infected group with BoHV-1, while the treated group with Artesunate showed a very minor pneumonic lesion. The negative control group of rabbits which was raised in the same conditions showed no lesions. These results are in agreement with Efferth et al. [15]. They mentioned that Artesunate was safer than primary extracts, Artemisinin, to be used on the experimental animals which did not show this toxicity on animals.

Artesunate inhibits numerous herpes viruses [15, 19, 20] and, this study now adds BoHV-1 to this list and gives initial results for the inhibition of BoHV-1 replication by ART. Besides, these results propose that Artesunate would be considered as a candidate for clinical use, especially for herpes viruses associated cases.

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