INVESTIGATION OF THE ANTIVIRAL EFFECT OF RIBAVIRIN AND ACYCLOVIR ON CANINE DISTEMPER AND INFECTIOUS CANINE HEPATITIS VIRUSES

El-Gallad, S.B.

Veterinary Serum and Vaccine Research Institute
Abbassia, Cairo, P.B.131

ABSTRACT

Investigating the cytotoxic and antiviral effects of Ribavirin on Vero cells, it was found that ribavirin is safe for Vero cells where neither growth inhibition nor cell toxicity have been recorded and a potent anti-canine distemper virus with all used concentrations (0.1, 1.5; 10; 100 and 200 µg/ml). It was found that all vaccinated puppies with canine distemper vaccine did not show any abnormal clinical signs except 3 of them showed slight rise in rectal temperature (38.5–39°C) on the 2nd day post vaccination returned to its normal level (37-37.5°C) by the treatment with a dose of 200µg of ribavirin injected intramuscularly for 3 successive days. Also it was noticed that treatment of experimentally infected puppies with canine distemper revealed that the dose of 10µg/animal did not cure the disease completely where 2 puppies had progressed clinical signs of canine distemper like the untreated one while 100 and 200µg/animal resulted in complete curing of infected animals.

In addition, in vitro studies revealed that all tested concentrations of Acyclovir were found to be safe for Vero cell showing no cell toxicity and did not affect the cell growth kinetics. On the other side it was noticed that complete antiviral effect of Acyclovir against CAV-1 was obtained by the use of concentrations of 200µg/ml/100 TCID₅₀ of the virus. Vaccination of puppies with the live attenuated canine hepatitis vaccine resulted in rise in rectal temperature, diarrhea and corneal opacity in 2 puppies while the other 8 vaccinated puppies did not show any abnormal clinical signs.

So it could be concluded that ribavirin and acyclovir could be used as anti-canine distemper and anti-canine hepatitis respectively either in cases of post vaccination or post infection.
INTRODUCTION

Canine distemper (CD) is a major pathogen in dogs and other wild carnivore with the highest fatality rate beside rabies (Apple and Montali, 1994). CD is caused by an RNA virus related to the family Paramyxoviridae. The disease has characterized by respiratory manifestations, diarrhea, and emaciation and usually ends with death. Nervous and cutaneous forms may be occurred. Stress concurrent illness or immunosuppression of currently vaccinated or contact dogs with diseased individuals could result in disease (Hader et al, 1991).

ICH was identified as specific viral disease of dogs caused by canine adenovirus-1 (CAV-1) as stated by Emary et al (1978) and Green (1998). It is characterized by fever, vomiting, diarrhea, and leucopenia and prolonged bleeding time (Housein, 2004). The disease is also known as canine adenovirus infection and canine contagious hepatitis (El-Sawalhy, 1997).

The causative virus of ICH is a double stranded DNA and its capsomers arranged in icosahedral symmetry with no envelope surrounding nucleocapsid (Buxton and Fraser, 1977). It was suggested that vaccination of dogs against CAV-1 with live attenuated vaccine may cause disease signs (Green, 1998).

It is an interesting subject to know some thing about antiviral drugs which developed progressively over the last few years. The list of antiviral agents approved for use by the US Food and Administration, includes amantadine, acyclovir and ribavirin (Griffin, 1995). Generally, antiviral agents inhibit steps in virus-specific replication.

Ribavirin (1-B-D-Ribofuranosyl-1,2,4-triazole-3,1 carboxamide) has been shown to exhibit a potent antiviral effect against many DNA and RNA viruses in vitro and in vivo (Sidwell et al, 1972 and 1979). The mode of antiviral activity of ribavirin could be explained by: (1) decreasing the intracellular pools of guanosine triphosphate, thereby, indirectly suppressing synthesis of viral RNA; (2) synthesis of RNA with abnormal 5 cap structures which in turn leads to inefficient translation of viral transcript; (3) direct suppression of viral polymerase activity (Baron, 1963).

Ribavirin has been used in clinical trials with promising results in treating measles (Mannon and Arroyo, 1977); hepatitis A (Galvao and Castro, 1975) and herpes virus type-2 (Sidwell et al, 1979); and in vitro studies against rinderpest (Mouaz et al, 1995) and Rift Valley Fever virus (Eman et al, 2001).

Acyclovir is a guanine analogue commonly used antiviral drug of low cytotoxicity and primarily used...
for treatment of herpes simplex virus infection (*Gertrude, 1988*). It contains only a partial nucleoside structure and the sugar ring is replaced by an open-chain structure. It is selectively converted into acyclo-guanosine monophosphate (acyclo-GMP) by viral thymidine kinase, which is far more effective (3000 times) in phosphorylation than cellular thymidine kinase. Subsequently, the monophosphate form is further phosphorylated into active triphosphate form, acyclo-guanosine triphosphate (acyclo-GTP), by cellular kinase. Acyclo-GTP is a very potent inhibitor of viral DNA polymerase; it has approximately 100 times greater affinity for viral than cellular polymerase. As a substrate, acyclo-GMP is incorporated into viral DNA, resulting in chain termination. It has also been shown that viral enzymes cannot remove acyclo-GMP from the chain, which results in inhibition of further activity of DNA polymerase. It was recorded that acyclovir reduced the titer of herpes virus type-1(it is a DNA virus) in Vero cells and in skin samples from experimentally infected mice (*Piret et al, 2000*).

The present work aims mainly to investigate the effect of Ribavirin and Acyclovir as antiviral drugs on canine distemper and infectious canine hepatitis viruses in vitro and in vivo studies.

**MATERIAL & METHODS**

**1-Puppies:**
Forty local breed puppies of about 3-5 months old were used in the present study. They were kept under daily clinical observations in special isolated kennels up to 21 days before the application of the experimental work. These puppies were screened using serum neutralization test and found to be free from canine distemper (CD) and infectious canine hepatitis (CAV-1) antibodies. They were divided into four groups each of ten puppies.

The first group was vaccinated with the locally prepared live attenuated canine distemper vaccine using a dose of $10^3\text{TCID}_{50}$/ animal injected subcutaneously according to *Guirguis et al (1991).*

The second group was vaccinated with the locally prepared live attenuated specific ICH vaccine at the dose of $10^3\text{TCID}_{50}$ for each animal injected subcutaneously according to *Khodeir et al (2003, II).*

The third group was experimentally infected with the virulent CD virus through the intranasal route using a dose of $10^5\text{ED}_{50}$/animal according to *Guirguis et al (1991).*

On starting of CD clinical signs, these puppies were subdivided into 4 sub-groups (3 animals / each of the first 3 subgroups and 1 puppy in the fourth one) where the first 3 subgroups were treated with Ribavirin using a dose of 10; 100 and
200µg/ animal injected intramuscularly respectively for 5 days while the last puppy was kept without treatment.

The fourth group was experimentally infected through the oronasal route using $10^5$ TCID$_{50}$ of the virulent virus (CAV-1) / animal according to Khodeir et al (2003, II).

On starting of the clinical signs of infectious canine hepatitis, these puppies were subdivided into 4 subgroups (3 animals / each of the first 3 subgroups and 1 puppy in the fourth one) where the first 3 subgroups were treated with Acyclovir using a dose of 10; 100 and 200µg/ animal injected intramuscularly respectively for 5 days while the last puppy was kept without treatment.

2-Vaccines:

Locally prepared on monolayer cell culture live attenuated specific canine distemper (CD) and infectious canine hepatitis (CAV-1) vaccines were supplied kindly by the Department of Pet Animal Vaccine Research, Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo. These vaccines were used to vaccinate experimental puppies in the present study. As these vaccines are live viruses they were also used to investigate the antiviral effect of Ribavirin and Acyclovir in vitro studies and serum neutralization test.

These viruses had titers of 6.5 and 5.8log$_{10}$ TCID$_{50}$/ml for CD and CAV-1 respectively.

3- Viruses:

Virulent local strains of canine distemper and infectious canine hepatitis (CAV– 1 – Abbassia/2002) (Khodeir et al, 2003-I) were used in experimental infection of puppies. These viruses were supplied by the same department.

4-Ribavirin:

Ribavirin (1-B-D-ribofuranosyl-1,2,4-Triazole-3-carbamide) was supplied kindly by Dr.Roberts (Viratek, Inc.USA). Ribavirin stock solution was prepared by dissolving 100mg in 10 of phosphate buffer saline (PBS) and sterilized by filtration through a 22um filter.

5-Acyclovir:

Acyclovir (90{(2-hydroxyethoxy) methyl} guanine) was obtained from Sigma Chemical Company (St. Louis, Mo.) The commercial acyclovir 5% injection was obtained from a local pharmacy.

6- Detection of the drug cytotoxicity:

Three day Vero cell cultures in micro-titer plates were used to detect the cytotoxicity of ribavirin and acyclovir where different concentrations of both drugs of 200, 100, 50, 10, 5, 1 and 0.1µg/ ml were prepared
in PBS. 25 µl of each drug concentration was inoculated in each of 5 wells of 96 well tissue culture plates. The test included normal cell as contol.

7-Detection of the in-vitro antiviral effect of ribavirin and acyclovir:

Vero cell culture plates were infected with CDV and other plates were infected with ICHV using 25 µl/well of100 TCID$_{50}$ of the used virus. After one hour allowed for virus adsorption, the cells was washed twice with PBS then each concentration of 25 µl of each drug concentration was added to each of 5 tissue culture wells with 150 µl of maintenance medium. The test included normal cells and untreated virus controls. All plates were subjected to daily microscopic examination.

RESULTS & DISCUSSION

The present work revealed that ribavirin is a safe for Vero cells where neither growth inhibition nor cell toxicity have been recorded and a potent anti-canine distemper virus with all used concentrations (Tables 1&2). These results agree with those of Mouaz et al., (1995) who concluded that ribavirin has no toxic effect on Vero and BK cell cultures and has an antiviral effect against rinderpest virus which is an RNA virus member in the same family Paramyxovirdae with canine distemper virus having the same general characters.

It was found that all vaccinated puppies with canine distemper vaccine did not show any abnormal clinical signs except 3 of them showed slight rise in rectal temperature (38.5-39°C) on the 2$^{nd}$ day post vaccination returned to its normal level (37-37.5 °C) by the treatment with a dose of 200µg of ribavirin injected intramuscularly for 3 successive days. Also it was noticed that treatment of experimentally infected puppies with canine distemper revealed that the dose of 10µg/animal did not cure the disease completely where 2 puppies had progressed clinical signs of canine distemper like the untreated one while 100 and 200µg/ animal resulted in complete curing of infected animals as shown in table (3). These findings come parallel to those obtained by Sidwell et al (1972 and 1979) and Eman et al (2001) presenting clues for the safety and efficacy of ribavirin as antiviral agent both in vitro and in vivo.

In addition, in vitro studies revealed that all tested concentrations of Acyclovir were found to be safe for Vero cell showing no cell toxicity and did not affect the cell growth kinetics. On the other side it was noticed that complete antiviral effect of Acyclovir against CAV-1 was obtained by the use of concentrations of 200µg/ml/100 TCID$_{50}$ of the virus. Similar res-
ults were obtained by *Gertrude (1988)* and *(Piret et al, 2000)* who tested the antiviral effect of Acyclovir against HV-1 (which is a DNA virus as CAV-1).

Vaccination of puppies with the live attenuated canine hepatitis vaccine resulted in rise in rectal temperature, diarrhea and corneal opacity in 2 puppies while the other 8 vaccinated puppies did not show any abnormal clinical signs. These observations agree the fact that such vaccine may results in that post vaccinal reaction in few cases as recorded by *Green (1998)* and *Tizard (2000)*. Treatment of post vaccination affected puppies as well as experimentally infected puppies showed that complete recovery of these animals was obtained by using a dose of 200µg of Acyclovir/ puppy injected intramuscularly for 5 successive days while untreated puppy showed typical signs of infectious canine hepatitis and died within 7 days post infection. These results come in complete agreement with those obtained using Acyclovir against HV-1 in skin samples from experimentally infected mice by *(Piret et al, 2000)*.

Finally it could be concluded that ribavirin and acyclovir could be used as anti-canine distemper and anti-canine hepatitis respectively either in cases of post vaccination or post infection.

Table (1): Cytotoxic effects of Ribavirin and Acyclovir on Vero cells.

<table>
<thead>
<tr>
<th>Concentrations of tested drug (µg/ml)</th>
<th>Cytotoxic effect on Vero cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>By Ribavirin</td>
</tr>
<tr>
<td></td>
<td>By Acyclovir</td>
</tr>
<tr>
<td>0.1</td>
<td>There was neither growth inhibition nor cell toxicity.</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>200.0</td>
<td></td>
</tr>
</tbody>
</table>
Table (2): In vitro antiviral effect of Ribavirin and Acyclovir.

<table>
<thead>
<tr>
<th>Concentrations of tested drug (µg/ml)</th>
<th>Loss of canine distemper virus titer (log_{10}/ml) by Ribavirin</th>
<th>Loss of canine hepatitis virus titer (log_{10}/ml) by Acyclovir</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1.0</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>5.0</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>10.0</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>100.0</td>
<td>6.5</td>
<td>5.2</td>
</tr>
<tr>
<td>200.0</td>
<td>6.5</td>
<td>5.8</td>
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Table (3): In vivo antiviral effect of ribavirin against canine distemper.

<table>
<thead>
<tr>
<th>The used dose of ribavirin (µg/ml) for each puppy</th>
<th>Number of treated infected puppies</th>
<th>Number of recovered puppies</th>
<th>Complete curing percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>3</td>
<td>1</td>
<td>33.3</td>
</tr>
<tr>
<td>100.0</td>
<td>3</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>200.0</td>
<td>3</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>0 (control)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table (4): In vivo antiviral effect of Acyclovir against canine hepatitis.

<table>
<thead>
<tr>
<th>The used dose of Acyclovir (µg/ml) for each puppy</th>
<th>Number of treated infected puppies</th>
<th>Number of recovered puppies</th>
<th>Complete curing percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100.0</td>
<td>3</td>
<td>2</td>
<td>66.6</td>
</tr>
<tr>
<td>200.0</td>
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<td>100</td>
</tr>
<tr>
<td>0 (control)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
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REFERENCES


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الملخص العربي

استبيان تأثير الريبافيرين والأسيكلوفير كمضادات للفيروسات على فيروسى الديسمبر والالتهاب الكبدى الوبائى في الكلاب

سيد بيومى خليل الجلاد
معهد بحوث الأمصال واللقاحات البيطرية
العباسية- القاهرة- ص.ب. 131

في هذا العمل تم التعرف على تأثير كل من الريبافيرين والأسيكلوفير على خلايا الزرع النسيجي لكلى القرد الأخضر الأفريقي المستمرة وكذلك تأثيرهما المضاد لفيروس حصبة الكلاب والالتهاب الكبدى الوبائى على التوالى في خلايا الزرع النسيجية المعدية بهذين الفيروسين. وقد تبين أن
كلا الدوائين غير سام للمزارع النسيجية بأي من التركيزات المستخدمة (100-2000 ميكروجرام / مل).

كما لوحظ أن أفضل تركيز يعطي 100% تأثير مضاد لفيروس حصبة الكلاب هو 200 ميكروجرام من الريبافيرين في حين أن مثل هذا التأثير يمكن الحصول عليه باستخدام 200 ميكروجرام من الأسيكلوفير ضد فيروس الالتهاب الكبدى الوبائي.

أمكن استخدام جرعة 200 ميكروجرام من كلا العقاقرين لكل جرو تحقن بالعضو لمدة خمسة أيام للحصول على شفاء كامل للحيوانات التي تظهر أعراض جانبية بعد التحصين باللقاحات الحية.

وعلى ذلك يمكن القول باستخدام الريبافيرين والأسيكلوفير لعلاج الآثار الجانبية أو الإصابة بحصبة الكلاب أو الالتهاب الكبدى الوبائي في الكلاب على التوالي.