IN VITRO AND *EX VIVO* EVALUATION OF THE VIRUCIDAL AND ANTIVIRAL CAPACITIES OF A BIOFLAVONOID AGAINST EQUINE HERPES VIRUS TYPE 1

Alexandra Scipioni, Tatiana Art, Lorène Dams, Brieuc de Moffarts, Pierre Lekeux and Etienne Thiry

This text is a non-specialist public version of a research study partially presented at the Hippos conference of 2008. (Scipioni et al, 2008)

The study was undertaken under the authority of Prof. Etienne Thiry (Department of Virology of the Faculty of Veterinary Medicine of Liège) and was conducted in cooperation with Dr. Tatiana Art (Department of Physiology of the Faculty of Veterinary Medicine of Liège). Its aim was the evaluation of the virucidal and antiviral capacities of a bioflavonoid.

This study was partially funded by TWYDIL®.

This study showed:

In vitro : : a significant virucidal activity of the bioflavonoid and some of its activity on the viral replication process that must still be confirmed by future investigations.

Ex vivo : following oral administration of this bioflavonoid, biological fluids showed significant virucidal activity.

INTRODUCTION

Relations between viral pathologies, inflammation and performance are frequently studied in humans in sports as well as in horses. We should bear in mind that respiratory system diseases are the second most common cause of poor sports performance for horses. Among these diseases, viral infections are a major problem (for further information about these infections, see the previous article, p 4 - 9) where they were dealt with in part. We will review briefly the implications of herpes virus type 1 (EHV-1).

EHV-1 (*Alphaherpesvirinae* family) is a major pathogenic virus in horses. It exists endemically in the world and causes abortion and myelo-encephalopathy; as for EHV-4, it causes rhinopneumonia. Though vaccines (and their combined protection against EHV-4) exist, horses are not fully protected against these diseases.

Many years ago, antiviral drugs were developed for the treatment of herpes virus infections in humans. Tests *in vitro* proved their efficiency against EHV-1 and were encouraging, but, unfortunately, results of tests *ex vivo* were disappointing. Accordingly, the research for chemicals able to help fight against viral infections in horses as well as pre-

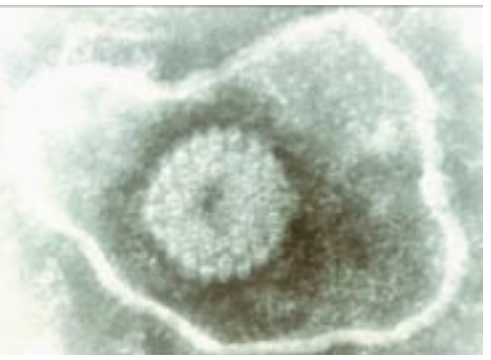


Figure 1 : Cell culture (RK13) infected by EHV-1 on decreased doses (left to right) (determination by $DICC_{50}$ method).

vent them became more and more intensive. So far, various plant extracts rich in flavonoids have shown some activity against human viruses (HIV, herpes etc.).

It should be remembered here that, whether clinical or subclinical, horse viral infections cause enormous economic losses that can even be catastrophic for the economy of a whole country (AQUIS report 2009).

PRESENTATION OF THE STUDY

The study took place in two phases: the first *in vitro* and the second *ex vivo*.

1. Study *in vitro*

The activity of flavonoids compound used on formulation of feed supplements was tested. Together with its effect on viral multiplication, its virucidal effect against EHV-1 was evaluated for an RK13 cell lineage based on a reading of the decrease of the viral titre.

This work was done in several stages:

- Producing a suspension of the compound using various solvents;
- Measuring the cytotoxicity of the solvents and of the compounds dissolved in an RK13 cell culture;
- Evaluation of the **virucidal effect** of the compounds. The principle was a measurment of the possible virucidal effect of the suspensions of flavonoids on EHV-1 in an RK13 cell culture. For this purpose, the sample was added to the viral suspension before infecting the cellular carpet. Different durations of contact (10 sec. to 30 min) were tested. The viral titre was measured after 3 days incubation using the method of infectious dose of the cells responsible for 50% lysis of the cell supports ($DICC_{50}$).
- Evaluation of the **compound's effect on viral multiplication:**

The objective was to measure the compound's effect on the multiplication process of the EHV-1 in an RK13 cell culture. The cells were brought into contact with the compound at different degrees of concentration in the course of the viral infection. Two days later, the culture boxes were examined for signs of cytopathogenic effects that would be characteristic of these viruses (syncytium and lysis zone).

2. Study *ex vivo*

In the second phase, the study aimed at showing a possible effect of the bioflavonoid in the plasma and/or the bronchoalveolar lavage (BAL) after 7 days oral administration of the flavonoid to 6 horses kept under standardized conditions.

In order to avoid interferences between the effect of the supplement and the reaction of potential antibodies against EHV-1 (there was a possibility that the horses might be already naturely immunized against this virus), porcine herpes virus 1 (SuHV-1) from the same family as EHV-1 was used for this phase of the test. All the experiments done with EHV-1 were validated by SuHV-1. Thus, only the effects of the flavonoid were taken into consideration, to the exclusion of possible antibodies that might be present.

Blood samples and bronchoalveolar lavages were carried out at three different times (D-1, D0 and D+7) and their virucidal effect tested.

The sampling was done following this plan:

- D-1: blood and BAL samples as control samples
- D0: blood sample (4 hours after the first administration)
- D+7: blood and BAL samples (4 hours after the last administration)

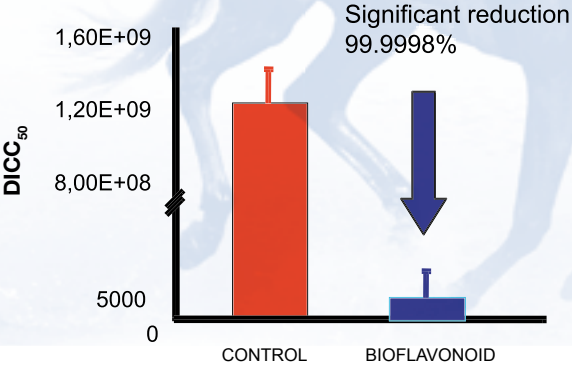


Figure 2 : viral count reduction after *in vitro* contact of the bioflavonoid with EHV-1

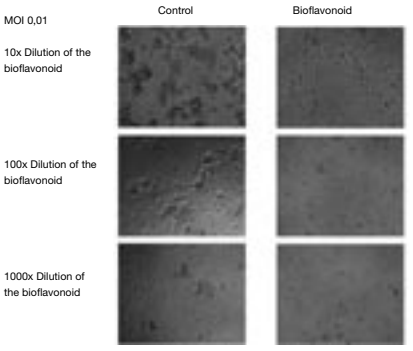
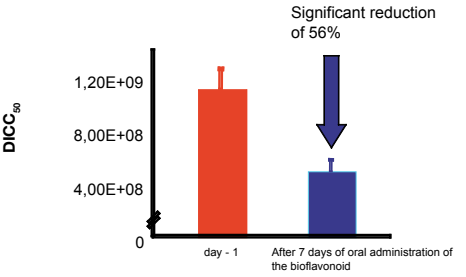


Figure 3 : observation after 24 hours of the cytophatogens effect induced in cells culture pre-incubated by EHV-1 without bioflavonoid. MOI : multiplicity of infection, representing of viral particuls per cells.

Figure 4 : viral count reduction after *ex vivo* contact of the broncho-alveolar lavage and EHV-1



The aim of the sampling was to answer the question whether – following the oral absorption –the flavonoid was still present in the horse's blood and/or BAL and whether it was available in a concentration that was enough for a virucidal effect *ex vivo*. The operation was in every point similar to the tests *in vitro* including the usual controls.

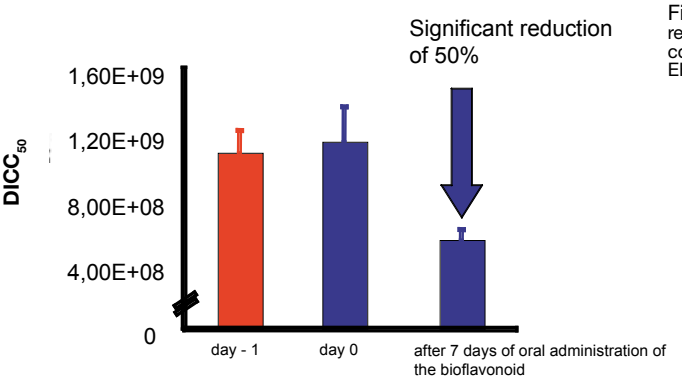


Figure 5 : viral count reduction after *ex vivo* contact of the plasma and EHV-1

RESULTS OF THE STUDY

1. Study *in vitro*

The verification revealed that the experimental conditions were appropriate. Figure 1 represents a reading plate for the EHV-1 in an RK13 cell culture after inoculation of the EHV-1 virus. The blue zones stand for cells still available and the white zones for lysed cells.

The virucidal effect of the compound on the EHV-1 proved significant! Just 10 seconds contact with a concentration of 0.125µg/ml was enough to reduce the viral titre by almost 100%. Figure 2 illustrates these results.

The outcome of the test suggests a possible effect of the compound on the multiplication process of the virus. Figure 3 indicates an absence of cytopathogenic reaction of the EHV-1 on RK13 culture cells when the bioflavonoid is added to the culture. However, the virucidal effect could interfere with the effect on the process of viral multiplication.

These results indicate clearly a virucidal effect and suggest an effect of the bioflavonoid on the viral multiplication process. Future additional studies must still investigate the latter in order to describe the corresponding behaviour.

2. Study *ex vivo*

Let us remember that the virucidal activity in the blood and the BAL was tested using the same methods as previously described, but by taking the porcine herpes virus 1 (SuHV-1) instead of EHV-1. The results of the readings were compared with those of the control. They are shown in figures 4 and 5. No significant virucidal effect could be demonstrated on D0 (i.e. 4 hrs after the first oral administration of the bioflavonoid). However, on D+7, a significant decrease of the viral titre was observed in the plasma as well as in the bronchoalveolar lavage.

In this phase of the study, the use of SuHV-1 helped us avoid a bias that could result from the presence of antibodies against EHV-1 in the horse. But another source of possible error is the presence of the EHV-1 virus itself! In this case, the virus could lyse the RK13 cells in its environment and thus lead to a false positive result. In order to eliminate this risk, the blood and BAL samples came first into contact with the RK13 cells without addition of the SuHV-1 virus. Subsequently, no cytopathogenic effect was observed, meaning that the animals were not in a phase of viral excretion.

The researchers also tried to measure the flavonoid's concentration in the physiologic fluids and, surprisingly, found that its concentration did not change significantly during the trial.

All these results suggest that the virucidal activity observed is due to the presence of the bioflavonoid's metabolites in the blood, since they had not been specifically looked for during the study. However, the activity of the biological fluids in the presence of EHV-1 following oral administration was observed.

GENERAL CONCLUSIONS

With this study, it could be demonstrated:

In vitro: that the bioflavonoid possesses a virucidal activity and that it probably has an effect on the process of viral multiplication.

Ex vivo: that, following oral administration of the bioflavonoid, some biological fluids have shown a significant virucidal activity.

QUESTIONS TO PROF. ETIENNE THIRY

HPH: Are these results surprising? Is this type of activity a frequent discovery?

Prof. E. Thiry: The observed virucidal effect *in vitro* is very important. Other studies showed this type of virucidal activity, but it is true that this type of effect had been mostly studied for bacteria and not for virus. These promising results allow us to expect an effect on horses infected by the EHV-1.

HPH: Some antiviral activity has been demonstrated, but what is the chemical's behaviour in your opinion?

Prof. E. Thiry: It is premature to specify the antiviral mechanisms of this molecule, because certain results must be confirmed. Regarding the virucidal effect, the inactivation of the viruses is probably due to an effect of the bioflavonoids on the envelope surrounding the virus.

HPH: Can this type of product be active against other viruses like, for example, the influenza virus or viruses in other animal species?

Prof. E. Thiry: If the action is located at the level of the viral envelope, it would be possible that the bioflavonoid has an effect on other wrapped viruses, such as the influenza viruses. Particular studies are however necessary to confirm this hypothesis.

HPH: Which perspectives can this type of research open?

Prof. E. Thiry: At the moment, vaccination is still the only form of prevention for viral diseases affecting horses. Thus, it could be usefully complemented by the use of virucidal bioflavonoids.

BIBLIOGRAPHY

Song J.M. and Seong B.L. Tea Catechins as a potential alternative anti-infectious agent. 2007. Expert Rev. Anti Infect. Ther., **5**; 497-506.

Whalley J. M., Robertson G. R., Scott N. A., Hudson G. L., Bell C. W. and Woodworth, L. M. Identification and nucleotide sequenceof a gene in equine herpesvirus 1 analogous to the herpes simplexvirus gene encoding the major envelope glycoprotein B. 1989. J. Gen. Virol. **70**; 383–394.

Scipioni A., Dams L., de Moffart B., Thiry E.,: In vitro susceptibility of equine herpesvirus type 1 to two feed additives containing flavonoids. In proceedings: Hippos congress, Liège, Belgium, 2008 (poster communication).

de la Fentes R., Awan A.R. and Field H.J. The acyclic nucleoside analogue penciclovir is a potent inhibitor of equine herpesvirus type 1 (EHV-1) in tissue culture and in murine model. 1992. Antiv. Res., **18**; 78-89.

Barrandeguy M., Vissani A., Ortiz C., Becerra L., Miño S., Pereda A., Oriol J., Thiry E.
Experimental reactivation of equid herpesvirus 3 following corticosteroid treatment. Equine Vet. J., 2008, **40**, doi: 10.2746/042516408X333399.

Fortier G., Pronost S., Miszczak F., Fortier C., Léon A., Richard E., Van Erck E., Thiry E., Lekeux P.
Identification of equid herpesvirus 5 in respiratory liquids: a retrospective study of 785 samples taken in 2006-2007. Vet. J., 2008, doi:10.1016/j.tvjl.2008.07.004.