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Virucidal activity of Nasaleze (Nasaval) and Nasaleze Travel (Nasaval Plus) in cell cultures infected with pathogenic avian flu virus (H5N1)

Lvov DK, Deryabin PG
Ivanovsky State Scientific Research Institute of Virology at the Russian Academy of Medical Sciences, Moscow.

Abstract

This *in vitro* study determined the viral efficacy of two cellulose formulations presented as Nasaleze (Nasaval) and Nasaleze Travel (Nasaval PLUS) against Influenza A/Duck/Novosibirsk/56/05 (Avian Flu H1N1) at concentrations that did not exhibit toxicity. Both test substances were used at sub-optimal dosing levels. The virucidal activity of both formulations was measured at 48, 72 and 112 hour periods after incubation. Results showed that both formulations were able to reduce the viral titre of Influenza A/Duck/Novosibirsk/56/05 (Avian Flu H1N1) significantly when compared to the control virus titre. The extract Nasaleze Travel (Nasaval PLUS) showed greater activity and both formulations showed potential to be used as preventative agents. These data reinforce the established antiviral activity of these formulations acting as barrier prevention and disruption of viral replication.

Introduction

In recent years a number of countries in East and South-East Asia have seen an outbreak of avian flu A (H5N1). The infection mainly affects poultry (chickens and ducks) which are then wiped out in their hundreds of thousands. But there have also been cases where the virus has affected people. The total number of people killed by the infection has been low but the fatality rate has been astonishing: around 70% of those infected have died, even when given treatment. The highly pathogenic avian flu virus arrived in Russia in July 2005 and to date the H5N1 flu virus has been recorded in many parts of the Russian Federation: in Western Siberia, in the Urals and in the Astrakhan province.

As we know, flu is primarily an infection which affects birds, mainly waterfowl, and all of the strains of the human flu virus come from avian bird flu viruses. The genome of any human virus contains genes from avian viruses.

Avian flu is extremely dangerous for humans, but fortunately it cannot be transmitted between people and can only be caught from infected birds. Human flu is easily transferred between people but the strains we are familiar with have become manageable on account of their joint evolution. However, some animals, primarily pigs, are easily infected with this and other types of flu. When the avian flu epizootic combines with a human flu epidemic (and they normally occur during the same months), both viruses can be found in pigs. The simultaneous reproduction of the two viruses in pigs may lead to reassortment and to the emergence of a new "hybrid" virus, in which the "avian" proteins and antigens of the avian flu A virus will combine with the ability to be transferred from person to person. At this point, a disaster is almost inevitable: the new agent will be infectious like human flu and lethal like bird flu. There is therefore a real threat of a new pandemic strain appearing.

We therefore need to develop new treatments and preventive measures for flu. At the D.I. Ivanovsky Scientific Research Institute of Virology we carry out research into the avian flu virus, developing diagnosis methods and treatment and preventive measures for the infection. Practically all known strains of avian and human flu viruses are held at the State Virus Collection at the Institute. It is precisely these viruses which could serve as the building blocks for a future pandemic virus. In particular, during the first outbreak of the H5N1 flu virus, we isolated the first highly pathogenic strains of this virus from patients and poultry (ducks and chickens) that had died from the disease, which were then deposited at the State Virus Collection. We are currently researching the decoding of the epizootics amongst birds in different parts of the country including the Republic of Kalmykia and the Astrakhan Province. Moreover, the research at the Institute is aimed at improving diagnosis methods, preventive measures and the treatment of this infection.

The D.I. Ivanovsky Scientific Research Institute of Virology at the Russian Academy of Medical Sciences, is licensed to carry out pre-clinical trials of different products, received commercial samples of two products to be studied from Pharmaval Inc. Nasaleze (Nasaval in Russia) and Nasaleze Travel (Nasaval PLUS in Russia), manufactured by Nasaleze Ltd, in Ramsey, Isle of Man. The aim of the research was to study the activity of these unique cellulose powder extracts against infection with the pandemic flu A/H5N1 virus in cell cultures, which we isolated during the poultry epizootic in July 2005 in the Novosibirsk province.

Materials and Methods

The virus

Our observations were carried out on both test substances and we determined the anti-viral activity against strains of the flu A/Duck/Novosibirsk/56/05 virus which was isolated in summer 2005 from infected ducks in the Novosibirsk province and deposited at the State Virus Collection. The virus multiplies in Madin-Darby canine kidney (MDCK) cell cultures (embryonic canine kidney cell cultures), in SPEV cell lines (porcine embryo kidney) and in many other cell cultures.

Cell cultures

Porcine embryo kidney cell cultures (SPEV) were used as the substrate for studying antiviral activity. This virus multiplies and accumulates in a titer of up to 4.5 lg TCD50 in these cultures. SPEV cell cultures were cultivated in medium 199 with the addition of 10% foetal bovine serum and antibiotics. As the support medium for the cells which have been infected with the flu virus we used the same nutrition medium composition without adding the serum. The cells were cultivated in single-use 24-hole sterile plastic culture plates. .

Test Samples

Nasaleze (Nasaval) and Nasaleze Travel (Nasaval PLUS) were used in the form of a ready-prepared nasal spray in 500mg bottles providing 200 doses, which we received from the Pharmaval Inc. During our trials we used one dose of each of the products which was the equivalent of one spray, equal to 2.5mg of the product.

Trial protocol
1st variant

On the second day after planting the SPEV cell cultures in 24-hole plastic plates, a cell monolayer had formed in the holes. The nutrient medium was removed from each of the holes, the holes were washed with 0.4 ml of the support medium, after which the holes were drained off, leaving around 0.1 ml of the medium in the hole. The spray containing each test substance was sprayed into each hole with a cell monolayer, with 1 spray of each of the products in 8 of the holes with the cell cultures. 10 minutes after the cells had been treated with the powder spray 20 µl of the flu A virus was added to 4 of the holes in a dose of 10.0 $TCID_{50}$, and 20 µl of the flu A virus was added to another 4 holes in a dose of 1.0 TCD_{50}. The 8 holes with a cell culture monolayer were infected with the flu virus in doses of 10.0 $TCID_{50}$ and 1.0 $TCID_{50}$ (4 holes for each dose), but were not treated with the products. The remaining 8 holes with a SPEV cell culture monolayer were not infected with the virus but were treated with the test substances in the same doses. After 30 minutes contact between the virus and the cells, 0.4 ml of the support medium (medium 199 with added antibiotics but without foetal bovine serum) was added to each of the holes and they were left in a germinator at 36.7° C. The percentage of healthy cells was determined towards the end of the experiment using methylene-blue.

2nd variant

On the second day after planting the SPEV cell cultures in 24-hole plastic plates, a cell monolayer had formed in the holes. The nutrient medium was removed from each of the holes, the holes were washed with 0.4 ml of the support medium, and the support medium was then drained off. Then 20 µl of the flu A virus was added to 8 holes in a dose of 10.0 $TCID_{50}$, and 20 µl of the flu A virus was added to another 8 holes in a dose of 1.0 $TCID_{50}$. After 30 minutes of contact for the virus to be adsorbed onto the cells, the powder spray containing Nasaleze (Nasaval) and Nasaleze Travel (Nasaval PLUS) was sprayed into each of the holes with the infected cell monolayer, with 1 spray of each of the products for the 4 holes with the cell cultures. The remaining 4 holes with the monolayer of infected SPEV cell cultures were not treated with the products. 0.4 ml of the support medium (medium 199 with added antibiotics but without foetal bovine serum) was then added to each of the holes and they were left in a germinator at 36.7° C. The infected cultures were observed over 4-5 days, and cytopathic changes were observed in the infected control cell cultures which were not treated with the test substances.

Determining the ability of the infected cells to produce the infectious flu A/H5N1 virus

48 hours after the cells were infected, 40 µL of the nutrient media was removed from the holes containing the infected SPEV cell cultures and the concentration of the infectious virus in the samples was determined through titration for infectious activity using a 2-day-old SPEV cell culture monolayer cultivated in 96-hole plates. After reaching the maximum display of cytopathic action, infectious titers were found in all of the test variants. The percentage of healthy cells was determined towards the end of the experiment using methylene-blue.

Results

The results are shown in tables 1 – 3.

Cytotoxic properties of the test substances

Upon visual observation under an optical microscope we were able to see that, in terms of morphological properties, vitality and cytoproliferative activity, the SPEV cell cultures did not differ from similar cells cultivated without treatment by the test substances over a period of 7-8 days' cultivation. On the first day after treatment with the test substances we were able to use the microscope to see a semi-transparent film covering the cell monolayer which disappeared after the 2nd day of observation and which had no effect on the vitality of the SPEV cells for the entire observation period.

Antiviral activity of Nasaleze (Nasaval) and Nasaleze Travel (Nasaval PLUS)

The information shown in table 1 shows that the test substances when treating the cell cultures before infection with the flu A/H1N1 virus (preventive application) in a dose of 2.5 mg per hole, are able to protect most of the

Table 1 : Antiviral properties of the products Nasaleze (Nasaval) and Nasaleze Travel (Nasaval PLUS) with regard to infection with the flu A/H5N1 virus in SPEV cell cultures. Effect on the vitality of infected cells when used for preventive purposes.

Dose of the virus (TCD50)	Products	Percentage of infected cells in the monolayer					
		SPEV+product+virus			SPEV without the product+virus		
		48 hours after infection	72 hours after infection	112 hours after infection	48 hours after infection	72 hours after infection	112 hours after infection
10.0	Nasaleze (Nasaval)	100±0	20±5	0	80±10	5±5	0
	Nasaleze Travel (Nasaval Plus)	100±0	75±10	0	80±10	5±5	0
1.0	Nasaleze (Nasaval)	100±0	85±10	0	95±15	30±5	0
	Nasaleze Travel (Nasaval Plus)	100±0	100±0	0	95±15	30±5	0

SPEV cell monolayer against the cytopathogenic effect of the flu A virus within 72 hours after infecting the cells. It was found that up to 85% - 100% of the cells in the monolayer survive when treated with the product Nasaleze Travel (Nasaval PLUS), while a total of 30% of the SPEV cells infected with the flu virus which are not treated with the product survive. It was also found that Nasaleze Travel (Nasaval PLUS) has a slightly greater antiviral effect than original Nasaleze (Nasaval).

At 112 hours after infection, most of the cells in the control and experimental test variants had been killed.

We received similar data when using the test substances immediately after infecting the SPEV cell cultures (table 2). We also found that this depended on the characteristics of the product which was used. So, when infecting the SPEV cells with the flu A virus in a dose of 10.0 $TCID_{50}$ under the effect of the test substance Nasaleze (Nasaval) at 72 hours after infection, 25% of the infected cells survived (in the control samples which were not treated with the product 5% of the cells survived in these conditions).

Table 2 : Antiviral properties of the products Nasaleze (Nasaval) and Nasaleze Travel (Nasaval PLUS) with regard to infection with the flu A/H5N1 virus in SPEV cell cultures. Effect on the vitality of infected cells when used for medical and preventive purposes.v

Dose of the virus (TCD50)	Products	Percentage of infected cells in the monolayer					
		SPEV+product+virus			SPEV without the product+virus		
		48 hours after infection	72 hours after infection	112 hours after infection	48 hours after infection	72 hours after infection	112 hours after infection
10.0	Nasaleze (Nasaval)	100±0	25±5	0	75±10	5±5	0
	Nasaleze Travel (Nasaval Plus)	100±0	80±10	0	85±10	5±5	0
1.0	Nasaleze (Nasaval)	100±0	85±10	0	95±15	25±5	0
	Nasaleze Travel (Nasaval Plus)	100±0	90±0	0	95±15	30±5	0

If the cell cultures were treated with the product Nasaleze Travel (Nasaval PLUS), 80% of the cells survived after 72 hours. However, in these conditions cells in all of the test variants had died at 112 hours after infection. Multiple treatments of the cells with the products would most probably be needed in order to achieve a stable antiviral effect.

It was interesting to learn about the effect of these test substances on the ability of the infected SPEV cells to produce the flu A virus in the medium. The results of titration of the samples of the medium collected from the infected cell cultures at 72 hours after infection are shown in table 3.

Table 3 : Antiviral properties of the products Nasaleze (Nasaval) and Nasaleze Travel (Nasaval PLUS) for the flu A/H5N1 virus in SPEV cell cultures. Effect on the concentration of the infectious virus produced by the cells (during preventive use of the products). Virus dose of 1.0 lg TCID50.

Route of administration	Products	Flu A virus titers (lg TCID50/ml) 72 hours after infection	
		SPEV+product+virus	SPEV without the product+virus
		72 hours after infection	72 hours after infection
Preventive	Nasaleze (Nasaval)	3.0±0.5	7.5±0.5
	Nasaleze Travel	1.5±0.5	7.5±0.5
Medical and preventive	Nasaleze (Nasaval)	4.0±0.5	7.5±0.5
	Nasaleze Travel (Nasaval Plus)	3.0±0.5	7.5±0.5

These show that at 72 hours after infection, the Nasaleze (Nasaval) test substance was able to reduce the production of the virus by the cells by 10,000+ times when compared with the production of the virus by untreated cells (table 3). In these conditions Nasaleze Travel (Nasaval PLUS) significantly reduced the infectious activity of the virus (to 6.0 lg $TCID_{50}$). Significant but somewhat lower levels of antiviral activity of the products were shown when using them for medical and preventive purposes (table 3).

These data sets indicate that the test substances Nasaleze (Nasaval) and Nasaleze Travel (Nasaval PLUS) are able to protect the cells from the cytopathogenic effect of the highly pathogenic flu A/H5N1 virus. The factors involved in the antiviral effect of theses natural compounds require further research. At the same time, we should point out the known viricidal qualities (ability to inactivate the infections properties of virions) of phytoncides in the composition of Nasaleze Travel (Nasaval PLUS) would suggest that it is superior to Nasaleze (Nasaval). However, the data generated clearly shows the antiviral effect of Nasaleze (Nasaval) without adding phytoncides. Here we should point out that the test substances, which are presented as microcellular powder, after treatment of the cell monolayer in combination with culture fluid, form a gel-like film layer which is often used in virological research to limit the reproduction of viruses. It is possible that this film may protect the cells against the adsorption of viruses onto their membrane.

Furthermore if the virus still penetrates the cell where it is not protected by the film, the virus which has multiplied and left the cell cannot be passed on to healthy cells which are protected by the film. Therefore, the infection process is significantly slowed down and could even be stopped with multiple applications of the test substances. It is also likely that the toxins and proteins which are formed as a result of the death of the infected cells will be used by the film, swept down into the stomach by normal muco-cilliary clearance mechanisms and will not cause intoxication or allergisation, which are observed during the normal infection process.

Conclusion and Discussion

The test substances Nasaleze (Nasaval) and Nasaleze Travel (Nasaval PLUS), provided by Pharmaval Inc, are able to protect most cell cultures from the cytopathogenic effect of the flu A/H5N1 virus. Our results indicate the Nasaleze Travel (Nasaval PLUS) product has more pronounced antiviral properties than the Nasaleze (Nasaval) formula. Both substances are however capable of significantly reducing the production of the flu A/H5N1 virus by infected cells over a period of 72 hours after the cells are infected using the equivalent of just 1 daily dose. Moreover, neither test substance showed any cytotoxic properties for SPEV cell cultures.

It is clear that these simple patented natural formulations have some interesting virucidal properties that warrant further investigation and that they could certainly be utilized as an alternative in preventing and perhaps treating active viral infections including the currently well described "avian flu". Our data indicate very strongly that Nasaleze (Nasaval) and particularly Nasaleze Travel (Nasaval PLUS) could be used both as a preventative measure and a treatment option for this pernicious and persistent viral infection.