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Effects of a nutrient mixture on infectious properties of the highly pathogenic strain of avian influenza virus A/H5N1

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Abstract. Numerous outbreaks of avian influenza virus infection (A/H5N1) have occurred recently, infecting domestic birds, chicken and ducks. The possibility of the emergence of a new strain of influenza virus capable of causing a pandemic in humans is high and no vaccine effective against such a strain currently exists. A unique nutrient mixture (NM), containing lysine, proline, ascorbic acid, green tea extract, N-acetyl cysteine, selenium among other micro nutrients, has been shown to exert a wide range of biochemical and pharmacological effects, including an inhibitory effect on replication of influenza virus and HIV. This prompted us to investigate the potential anti-viral activity of a nutrient mixture (NM) and its components on avian influenza virus A/H5N1at viral dosages of 1.0, 0.1 and 0.01 $TCID_{50}$. Antiviral activity was studied in cultured cell lines PK, BHK-21, and Vero-E6. Virus lysing activity was determined by co-incubation of virus A/H5N1 with NM for 0–60 min, followed residual virulence titration in cultured SPEV or BHK-21 cells. NM demonstrated high antiviral activity evident even at prolonged periods after infection. NM antiviral properties were comparable to those of conventional drugs (amantadine and oseltamivir); however, NM had the advantage of affecting viral replication at the late stages of the infection process.

Keywords: Nutrient mixture, avian influenza virus A/H5N1, cell cultures, cytotoxicity, antiviral activity survival

1. Introduction

Since 2002, influenza A H5N1 viruses originating in Asia in wild bird species [6] have spread across Asia, Europe, the Middle East and some African countries infecting additional species such as tigers, leopards, cats, stone martens and humans [39]. Incidence of avian influenza virus infections has increased in the last decade, occurring in association with poultry outbreaks; incidence of influenza A HPAI H5N1 surfaced in December of 2003 in Korea and China in poultry, and the outbreak has since stretched worldwide. Human cases followed the poultry outbreak with a total of 335 cases and 206 fatalities as of the end of 2007 [40]. Since July 2005, a highly pathogenic strain of avian influenza virus has invaded Russian territories. Since then, infection with influenza virus H5N1 has been registered in many

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regions, including: Western Siberia, the Ural Mountains, the Astrakchan region, and the Republic of Tyva [22–25].

Wild birds (ducks, geese, swans and shorebirds) are natural reservoirs of influenza viruses, and all of the known 16 HA and 9 NA subtypes have been found in these birds. All strains of human influenza virus have eventually evolved from bird virus, and the genome of any human strain virus shares genes with avian strains [3]. Though avian influenza virus is rarely transmitted from person to person, human influenza virus is very easily transferable from person to person; however, currently known strains are contained by co-evolution: point mutation (antigenic drift) and RNA segment reassortment (antigenic shift) [20,21].

The hemagglutinin (HA) glycoprotein mediates attachment and entry of the virus by binding to sialic acid receptors on the cell surface. The binding affinity of the HA to the host sialic acid allows for the host specificity of influenza A [8,15]. Avian influenza subtypes prefer to bind to sialic acid by α-2,3 linkages, while human subtypes bind to those with α-2,6 linkages. Swine, however, easily catch both influenza viruses since they contain both types of linkages. When outbreaks of avian influenza coincide with outbreaks of human influenza (both tend to occur during the same seasons) both viruses could be found in swine. Simultaneous reproduction of two different viruses in one body could lead to their re-association and evolution of a new hybrid virus capable of transferring avian flu virus protein-antigens between humans. Such an event could prove to be catastrophic since the new virus would be as infectious as a human one and as deadly as an avian one. Thus, the possibility of the appearance of a new pandemic strain of influenza virus is quite real.

The World Health Organization (WHO) predicts a high probability of a new influenza virus with pandemic properties emerging as soon as the next several years. No vaccine capable of containing such an epidemic is available and, at the moment, there is no domestic production of conventional potent anti-influenza medication in Russia. This situation dictates an urgent effort in the search for new ways of influenza treatment and prevention.

D.I. Ivanovsky Research Institute on Virology (DIIRIV) conducts research on avian influenza virus including diagnostics, treatment and prevention of the infection. The State Virus Collection at DIIRIV preserves practically all currently known strains of human and avian influenza viruses, including those capable of evolving to a pandemic strain. In particular, during the first outbreak of H5N1 virus in Russia in July 2005, the first highly pathogenic strains of this virus were obtained from affected domestic birds and deposited into the State Virus Collection. Biological properties of the isolated virus strains were investigated and experimental data on their sensitivity to conventional anti-influenza medications (rimantadine, arbidole, oseltamivir) were obtained [5,12,18,25]. Research on deciphering the disease epizoology among birds in other regions enabled us to isolate highly pathogenic strains of avian influenza virus from swans in Astrakchanj region, wild birds in the Republic of Tyva and others.

Dr. Rath Research Institute has focused on developing strategies to inhibit cancer development, heart diseases, viral infections and other diseases by naturally occurring nutrients. Previous research studies have demonstrated that a nutrient mixture (NM) containing lysine, proline, ascorbic acid, green tea extract, N-acetyl cysteine (NAC), selenium and other micro nutrients has potent anti-carcinogenic, anti-atherogenic and antiviral activity both *in vitro* and *in vivo* [16,34]. Since some viruses can spread in the body using similar mechanisms as cancer, such as production of MMPs in influenza virus-infected cells as demonstrated by Yeo et al. [41], we postulated that this relatively non-toxic nutrient mixture would have an effect on influenza viral infections in host cells.

Literature has reported many of the constituents of NM to have an inhibitory effect on replication of influenza virus and other viruses [2,7,11,13,14,31,37,38]. This prompted us to investigate the anti-viral activity of a nutrient mixture (NM) on the highly pathogenic strain of influenza virus type A/H5N1 in an experimental model employing cultured animal cells of different origin.

2. Materials and methods

2.1. Virus

Avian influenza virus type A/H5N1 utilized in the study was isolated from domestic ducks during an influenza epizooty in Novosibirsk region in July 2005 [24] and was deposited in the state collection of virus strains as A/duck/Novosibirst56/05. Stock virus preparations in conditioned cultured cell medium were collected from cultured porcine embryonic kidney cells (SPEV) infected with influenza virus type A/H5N1 at a peak of cytopathological events. The infectious $TCID_{50}$ titer of stock virus preparations used in this study were 6.5–7.0 $\log_{10}$. In cell experiments A/H5N1 virus dosages were 1.0, 0.1 and 0.01 $TCID_{50}$.

2.2. Cell cultures

Antiviral activity was studied in cultured cell lines PK (pig kidney cells), BHK-21 (Syrian hamster kidney), and Vero-E6 (green monkey kidney), sensitive to infection with A/H5N1 virus [4], seeded for 24 h to confluent density in plastic 48-well plates in 199 medium (SPEV) or Eagle's medium (BHK-21 and Vero-E6), (supplied by the Institute of Poliomyelitis and Encephalitis, Russian Academy of Medical Sciences) supplemented with 10% fetal bovine serum (FBS, HyClone), antibiotics (100 U/ml penicillin and streptomycin) and glutamine. Cell cultures were infected with virus by standard protocol and cultivated using the same medium but without FBS. Virus infectious titer was calculated according to Reed and Muench [33].

2.3. Nutrient Mixture (NM) stock solution

The nutrient mixture (NM) powder was composed of the following in the ratio indicated: Vitamin C (as ascorbic acid and as Mg, Ca, and palmityol ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 μg; copper 2 mg; manganese 1 mg. The powder was kept in a plastic tube at 4 °C in the dark for less than a month. A stock solution of 5 mg/ml NM was prepared daily by dissolving it in serum-free cell culture medium while vortexing for 60 sec at 25°C, followed by filtration through sterile 0.22-micron filter. The stock solution was kept at 4°C and used within 30 min after preparation. Stock solution of NM was diluted with corresponding cell culture media to working concentrations of 125, 250 or 500 μg/ml, of which 50 μl was added to experimental wells. Individual components of NM were diluted to 5 mg/ml in 199 medium (stock solutions). For experiments, stock solutions were diluted 1 to 10 (500 μg/ml) or 1 to 20 (250 μg/ml) with cell culture medium and 50 μl of it was added to 100 μl of cell culture media in wells.

2.4. Cytoxicity of the Nutrient Mixture (NM)

NM cytoxicity was studied in Vero E6 cell cultures. The NM stock solution 5 mg/ml was sequentially titrated 1:1 with Eagles medium and 50 μl from the resulting solution was added to Vero E6 cell suspension cultures. Trypan blue exclusion test was run after three days incubation.

2.5. Virocidal activity

Virus lysing activity was determined by co-incubation of the virus containing media with nontoxic concentrations of NM (1:1, v:v) for 0, 10, 30 and 60 min, followed by virus cytopathogenic activity assay by residual virulence titration in PK or BHK-21 cell cultures.

Table 1A
Cytotoxic properties of NM in cultures of suspended Vero-E6 cells

Treatment	Cell Survival rate (%) after treatment with a dilution indicated					
	1:2	1:4	1:8	1:10	1:20	Virus only Control
100 μl NM	0	0	20	60	80	100
50 μl NM	0	0	60	100	100	100
Virus only Control	100	100	100	100	100	100

Pre-formed cell monolayer was completely restored in 3–4 days after addition of 100 μl of NM in dilutions 1:10 and 1:20. Cytotoxic effect was evident by cell rounding and/or detachment.

Table 1B
Cytotoxic properties of NM in cultures of attached Vero-E6 cells (2 day monolayer)

Treatment	Cell Survival rate (%) after treatment with a dilution indicated					
	1:2	1:4	1:8	1:10	1:20	Virus only Control
100 μl NM	0	0	60	100	100	100
50 μl NM	0	0	60	100	100	100
Virus only Control	100	100	100	100	100	100

2.6. Antiviral activity

Antiviral activity of NM and its components was assayed following the guidelines of the Russian Pharmacology Committee [27]. Cell monolayer survival rate after viral infection was determined by Trypan Blue exclusion test in monolayer of viable cell cultures 72 h after infection. Effects on cell capacity for viral reproduction were determined by residual virulence titration of conditioned cell culture medium collected 24 h after viral infection.

2.7. Statistical analysis

The results for each study are expressed as means ± and standard errors, derived from three or four experiments.

3. Results and discussion

3.1. Cytotoxicity of the nutrient mixture

As shown in Table 1A, NM had dose-dependent cytotoxic effects on cultured Vero-E6 cells. Addition of 100 μl NM diluted 1:2 to 1:8 to freshly prepared cell suspensions resulted in 100% cell death, whereas addition of NM dilutions 1:10 and 1:20 caused 40% and 20% cell death, respectively. Addition of 50 μl of NM solution diluted 1:10 and 1:20 resulted in 100% cell survival. When the cell monolayer was tested 24 h after seeding, similar dosages of NM produced no cytotoxic effect even when added at the volume of 100 μl, as shown in Table 1B. Only non-toxic dosages of NM were used in subsequent experiments.

3.2. Effect of NM on virulence of H5N1 strain virus

NM was shown to have high virolytic properties on avian flu virus A/H5N1, as shown in Table 2. NM reduced virulence to 3.01 g after virus pre-treatment with NM for 30 min and 60 min. NM reduced H5N1 virulence by 10 and 100 fold after virus treatment for a few seconds. NM at dilutions 1:10 and 1:20 effectively inhibited viral infection activity after treatment during 10 to 60 min (Table 2).

Table 2
Virolytic properties of NM on infectious capacity of avian influenza virus type A H5N1

Virus exposure time with NM	A/H5N1 Virus titer as tested in cultured cells (log_{10} $TCID_{50}$)			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
0 min	5.0	5.5	ND	2.0
10 min	5.1	5.0	1.5	0
30 min	4.8	4.5	2.0	0
60 min	3.0	3.5	1.7	0
Control (Untreated)	6.0	7.5	4.1	3.8

Table 3A
Anti-viral effects of NM against Vero-E6 cell infection with avian influenza virus type A H5N1. Cell survival at indicated time points after viral infection following 1 h pre-incubation with NM (μg/ml). NM remained in cell culture media to the end of experiment

Viral load (log_{10} $TCID_{50}$units)	Cell Survival (%)								
	500 μg/ml NM			250 μg/ml NM			Control (Virus only)		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1.0	100	100	100	100	100	100	25	0	0
0.1	100	100	100	100	100	100	50	50	25
0.01	100	100	100	100	100	100	90	85	45

Table 3B
Anti-viral effects of NM against BHK-21 cell infection with avian influenza virus type A H5N1. Cell survival at indicated time points after viral infection following 1 h pre-incubation with NM (μg/ml). NM remained in cell culture media to the end of experiment

Viral load (log_{10} $TCID_{50}$units)	Cell Survival (%)								
	500 μg/ml			250 μg/ml			Virus only Control		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1.0	85	50	25	50	50	25	25	0	0
0.1	100	50	100	100	90	85	50	50	25
0.01	100	100	100	100	100	100	90	85	45

Table 3C
Anti-viral effects of NM against PK cell infection with avian influenza virus type A H5N1. Cell survival at indicated time points after viral infection following 1 h pre-incubation with NM. NM remained in cell culture media to the end of experiment

Viral load (log_{10} $TCID_{50}$units)	Cell Survival (%)								
	NM 500 μg/ml			NM 250 μg/ml			Virus only Control		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1.0	50	50	25	50	25	25	25	0	0
0.1	100	100	50	100	100	100	40	50	25
0.01	100	100	90	100	100	100	75	85	45

3.3. Antiviral effect of NM on PK, BHK-21 and vero E6 cells infected with H5N1 strain virus

To investigate involvement of other mechanisms in NM antiviral activity, NM was added to PK, BHK-21 and Vero-E6 cell cultures: prior to addition of virus; simultaneously with addition of virus; or after cell exposure to viral infection. As shown in Table 3A, treatment of Vero-E6 cell cultures with NM at dosages 500 and 250 μg/ml for 1 h prior to viral infection demonstrated 100% protective effect, while addition of the same virus dosage to control cells (untreated with NM), caused 100% cell death at 72 h. Similar results were observed when lower dosages of avian flu virus type A/H5N1 were used. Similar results were observed when cells were treated with NM simultaneously with viral infection (Table 3B).

Table 4
Anti-viral effects of NM against BHK-21 cell infection with avian influenza virus type A/H5N1. Cell survival at 72 h after viral infection following pre-incubation with NM. Cells were pre-treated with NM for indicated period of time before infection. NM was removed from cell culture media immediately before infection with virus

Incubation time with NM	NM (μg/ml)	72 h cell survival rate (%) after infection with virus in indicated dosages ($\log_{10}$ $TCID_{50}$)		
		1.0	0.1	0.01
1 h before infection	500	50	100	100
	250	25	30	25
	125	0	0	0
3 h before infection	500	50	100	100
	250	25	25	25
6 h before infection	500	0	0	100
	250	0	0	100
24 h before infection	500	0	0	25
	250	0	0	25
Virus only (Control)	0	0	0	25

NM completely protected SPEV cell cultures from infection with a 0.1 $TCID_{50}$ viral dose, whereas 75% of control cells died in 72 h. Cell cultures were partially protected by NM even at a higher viral load (1.0 $TCID_{50}$). Results listed in Table 3C suggest that NM used at concentrations 500 and 250 μg/ml protected most SPEV cell cultures from pathogenic viral activity when viral load was 0.1 and 0.01 $TCID_{50}$. When the viral load was increased to 1.0 $TCID_{50}$, cell survival in NM-treated cell cultures was 25%, whereas it caused 100% cell death in control cell cultures.

3.4. Optimal time of cell pretreatment with NM prior to viral infection

We were interested in determining the optimal time of cell pre-treatment with NM prior to application of viral infection and if the effects of NM would be altered if it were removed from cell culture medium prior to viral infection. Results listed in Table 4 suggest that cell pre-treatment with NM for 24 h or 6 h before viral infection did not cause significant increase in cell survival, except in cells treated with a viral load of 0.01 $TCID_{50}$, when 6 h pre-treatment with NM led to 100% cell survival. NM dosages of 500 and 250 μg/ml effectively protected cell viability against a viral load of 0.1 and 0.01 $TCID_{50}$ (Table 4).

Results of the experiments on titration of conditioned cell culture medium collected from BHK-21 cell cultures 24 h after viral infection, as shown in Table 5, confirmed antiviral effects of NM. Treatment with NM caused significant decrease of viral content in cell culture media of BHK-21 cell cultures infected with 0.01 $TCID_{50}$ viral load. However, at the higher viral load, antiviral effects of NM were not evident when applied 6 h before viral infection. Still, viral content of cell conditioned media decreased to zero when 500 μg/ml NM was applied 1 h and 3 h before infection. NM applied at 250 μg/ml did not produce an antiviral effect when the viral load was 1.0 $TCID_{50}$ (Table 5).

As evident from the experiments of viral infection of BHK-21 cell cultures, maximal antiviral effects of NM developed when NM was applied at the dosages of 250 and 500 μg/ml simultaneously with viral infection or following it, as shown in Table 6. When cells were infected with the viral load at 0.1 $TCID_{50}$, 72 h cell survival rate was 100% if NM was applied simultaneously with virus, or 1h or 24 h later. In cell cultures not treated, a similar viral load caused 100% cell mortality in 72 h. The viral titer in control cell cultures infected with 0.1 $TCID_{50}$ reached values of 5.2 ($\log_{10}$) $TCID_{50}$, whereas no infectious virus could be found in cell cultures treated with NM (at dosages 250 and 500 μg/ml) 1 h or 24 h after viral infection (Table 7).

Table 5
Effects of BHK-21 cell pre-incubation with NM before viral infection on virus content in cell conditioned media assayed 24 h after viral infection

Incubation time with NM	NM μg/ml	Conditioned media virus titer (log_{10} $TCID_{50}$) in 24 h after infection		
		1.0	0.1	0.01
1 h before infection	500	0	0	0
	250	4.1	0	0
	125	5.0	4.0	3.8
3 h before infection	500	0	0	0
	250	4.1	2.0	1.2
6 h before infection	500	3.0	3.0	0
	250	4.0	4.0	0
Virus only Control	0	5.2	3.6	2.7

Table 6
Antiviral effects of NM on BHK-21 cell culture survival rate after infection with avian influenza virus type A H5N1. Cells were treated with NM simultaneously with viral load or followed infection by indicated period of time

Time of NM addition	NM (μg/ml)	72 h cell survival rate (%) after infection with 0.1 $TCID_{50}$ (log_{10})
Simultaneously with Virus	500	100
	250	100
	125	90
1 h after infection	500	100
	250	100
	125	100
24 h after infection	500	100
	250	100
	125	100
Virus only Control	0	0

Table 7
Antiviral effects of NM on BHK-21 cell conditioned media virus titer after infection with avian influenza virus type A H5N1. Cells were treated with NM simultaneously with viral load or followed infection by indicated period of time

Time of NM addition	NM, μg/ml	Conditioned media virus titer (log_{10} $TCID_{50}$) in 24 h after infection
Simultaneously with Virus	500	0
	250	0
	125	2.0
1 h after infection	500	0
	250	0
	125	0
24 h after infection	500	0
	250	0
	125	0
Virus only Control	0	5.2

Statistical analysis of the data, listed in Tables 8 and 9, confirm antiviral effects of NM, which are characterized by preventive and, even more pronounced, therapeutic activity. The therapeutic activity of NM was evident even 24 h after infection, as shown in Table 9.

Table 8
Antiviral activity of NM in the experimental model of PK cell culture infected with avian influenza virus type A H5N1 at the dose of 0.1 $TCID_{50}$ (log_{10}). Cell Survival Rate. Results of statistical analysis

Time of NM addition	PK Cell Survival Rate in 72 h after infection				
	NM 1000 μg/ml	NM 500 μg/ml	NM 250 μg/ml	NM 125 μg/ml	Virus only Control
24 h before infection	75 ± 10	70 ± 5	0	0	0
1 h before infection	75 ± 10	75 ± 10	50 ± 10	0	0
Simultaneously with infection	90 ± 10	95 ± 5	90 ± 10	95 ± 5	0
1 h after infection	95 ± 5	95 ± 5	85 ± 15	90 ± 10	
4 h after infection	90 ± 10	95 ± 5	90 ± 10	95 ± 5	0
24 h after infection	90 ± 10	85 ± 15	80 ± 15	0	0

Table 9
Antiviral activity of NM in the experimental model of PK cell culture infected with avian influenza virus type A/H5N1 at the dose of 0.1 $TCID_{50}$ (log_{10}), Infectious virus titer (log_{10} $TCID_{50}$) in cell conditioned media. Results of statistical analysis

Time of NM addition	Infectious virus type A/H5N1 titer (log_{10} $TCID_{50}$) in PK cells conditioned media 24 h after infection				
	NM 1000 μg/ml	NM 500 μg/ml	NM 250 μg/ml	NM 125 μg/ml	Virus only Control
24 h before infection	0	0	2.2 ± 0.4	4.2 ± 0.5	4.5 ± 0.5
1 h before infection	0	0	0	0	4.5 ± 0.5
Simultaneously with infection	0	0	0	0	4.5 ± 0.5
1 h after infection	0	0	0	0	4.5 ± 0.5
4 h after infection	0	0	0	0	4.5 ± 0.5
24 h after infection	0	0	0	5.0 ± 0.5	4.5 ± 0.5

Table 10
Comparison of antiviral activity of NM to conventional antiflu medicines in PK cells infected with avian influenza virus type A/H5N1 at viral load of 0.01 $TCID_{50}$ (log_{10}). Cell Survival Rate

Time of addition of tested compound	Survival rate (%) of PK cells at 72 h after infection with viral load of 0.01 $TCID_{50}$ (log_{10})								
	NM μg/ml		TamiFlu, μg/ml			Amantadine, μg/ml			Virus only Control
	500	250	100	50	25	100	10	5	
1 h before infection	100	25	toxic	100	100	toxic	100	100	25
Simultaneously with infection	100	100	toxic	100	100	toxic	100	100	25
1 h after infection	100	100	toxic	100	100	toxic	100	100	25
2 h after infection	100	100	toxic	75	50	toxic	50	25	25

3.5. *Comparison of antiviral activity of NM with oseltamivir and amantadine in infected vero cells*

A comparative analysis of antiviral activity of NM and conventional anti-flu medicines (amantadine and oseltamivir) was performed, as shown in Tables 10 and 11. Oseltamivir was cytotoxic at 100 μg/m, with reduced cytotoxic effects at 50 and 25 μg/ml. When used at non-toxic dosages 1 h before, 1 h after or simultaneously with viral infection, oseltamivir prevented death of all infected cells. In contrast to NM, oseltamivir had significantly lower antiviral effects at later stages of infection (2 h after infection). Similar results were obtained in Vero-E6 and SPEV cell cultures.

Amantadine demonstrated antiviral activity against infection with A/H5N1 similar to that of oseltamivir. Similarly, its antiviral therapeutic activity was significantly reduced when applied at the late stages of viral infection (Tables 10 and 11). In contrast, NM demonstrated a high capacity to inhibit viral infection even when applied 24 h after infection.

Table 11
Comparison of antiviral activity of NM to conventional antiflu medications in SPEV cells infected with avian influenza virus type A H5N1 at viral load of 0.1 $TCID_{50}$ (log_{10}). Cell Survival Rate

Time of tested compound addition	Survival rate (%) of Vero-E6 cells at 72 h after infection with viral load of 0.1 $TCID_{50}$ (log_{10})					
	NM μg/ml		TamiFlu, μg/ml	Amantadine, μg/ml		Virus only Control
	500	250	50	10	5	
1 h before infection	95	50	90	95	70	20
Simultaneously with infection	85	75	100	95	100	20
1 h after infection	90	80	100	97	70	20
2 h after infection	90	85	50	45	20	20

Table 12
Effects of NM components on BHK-21 cell survival rate after infection with avian influenza virus type A/H5N1

Component	Dilution	Survival rate (%) of BHK-21 cells at 72 h after infection with indicated viral load (log_{10} $TCID_{50}$)			
		Component addition simultaneously with infection		Component addition 1 h after infection	
		0.1 $TCID_{50}$	0.01 $TCID_{50}$	0.1 $TCID_{50}$	0.01 $TCID_{50}$
Ascorbic Acid	1:10	10	50	0	90
	1:20	0	60	10	100
L-Lysine	1:10	10	95		
	1:20	10	60		
L-Proline	1:10	10	95	0	0
	1:20	0	15		
L-Arginine	1:10	0	10	0	0
	1:20	0	20		
N-acetyl cysteine	1:10	0	70	0	0
	1:20	0	50		
Green Tea Extract	1:10	100	100	100	100
	1:20	100	100		
NM	1:10	50	100	60	100
	1:20	20	75		
Virus only Control		0	40	0	40

3.6. *Antiviral activity of NM components*

Antiviral activity of individual NM components were tested utilizing the experimental conditions that yielded the most pronounced antiviral effects of NM against cultured cell infection with avian influenza virus type A/H5N1 when applied simultaneously or after infection (Tables 12 and 13). The highest antiviral activity was associated with green tea extract and NM itself, and much less with ascorbic acid, lysine and proline using a viral load of 0.1 TDC50. At a lower viral load, 75% cells were rescued by N-acetyl cysteine, 95% by lysine and proline, and 100% by green tea extract. Arginine at the dosage used did not provide any noticeable antiviral effect against influenza virus infection. Results from viral propagation studies in BHK-21 infected cell cultures, assayed in 24 h conditioned media, confirmed results obtained in cell survival experiments. As shown in Table 13, maximum antiviral activity was associated with green tea extract and NM, with lower effects observed with lysine and proline. Arginine at the dosages used did not reveal any antiviral activity, but supported virus reproduction to a certain degree. These results followed the general pattern of those observed in experiments with cell survival assay (Table 12).

Table 13
Effects of NM components on infectious virus titer in BHK-21 cell culture conditioned media in 24 hours after infection with avian influenza virus type A/H5N1

Component	Dilution	Infectious virus type A/H5N1 titer (log_{10} $TCID_{50}$) in cell conditioned media 24 h after infection with virus load of 0.1 $TCID_{50}$ (log_{10})	
		0.1 $TCID_{50}$	Changes against virus only (Control)
Ascorbic Acid	1:10	4.2	0
	1:20	4.2	0
L-Lysine	1:10	3.0	<1.2
	1:20	3.1	<1.1
L-Proline	1:10	3.0	<1.2
	1:20	3.1	<1.1
L-Arginine	1:10	5.0	>0.8
	1:20	5.2	>1.0
N-acetyl cysteine	1:10	4.1	<0.1
	1:20	4.2	0
Green Tea Extract	1:10	1.8	>2.4
	1:20	0	>4.2
NM	1:10	2.3	>1.9
	1:20	3.0	>1.2
Virus only Control		4.2	0

4. Discussion

In this study, NM demonstrated virolytic properties against avian influenza virus type A/H5N1 and a high level of antiviral activity against infection by the virus in a variety of mammalian cultured cells (SPEV, BHK-21 and VeroE6). NM demonstrated preventive and, even more pronounced, therapeutic antiviral properties even at later stages of viral infection.

Standard treatment of influenza infections in humans has included antiviral agents, such as the adamantanes (rimantadine and amantadine) and neuraminidase inhibitors (oseltamivir and zanamivir). However, these drugs have been associated with severe toxic side effects and with drug resistant variants, suggesting the need for improved therapies [29,30]. In our study, antiviral activity of NM was found to be comparable to that of conventional anti flu drugs amantadine and oseltamivir. However, NM also controlled viral infection at later stages of infection as compared to tested conventional drugs. Furthermore, NM was not found to be cytotoxic toward cultured cells used in the study when applied at concentrations up to 500 μg/ml. The nutrient mixture has been shown in our previous studies to inhibit influenza A neuraminidase activity and virus NP antigen production [17]. Furthermore, this nutrient mixture was shown to inhibit pathological effects in chickens resulting from avian flu [1].

The nutrient mixture was formulated based on targeting different physiological processes involved in cancer and infectious disease at the cellular level. A key component of NM is green tea extract, which is enriched in polyphenols such as EGCG. Green tea extract has demonstrated significant suppression of angiogenesis, metastasis, and other parameters of cancer progression [35]. Furthermore, EGCG was found have potent antiviral effects on influenza virus subtypes, inhibiting virus replication and RNA synthesis in MDCK cell cultures [37] and agglutination of influenza virus in cell culture, preventing viruses from absorbing to MDCK cells [31]. In testing NM components on avian influenza virus A/H5N1, the highest antiviral activity was associated with green tea extract, which was slightly greater than that shown by NM. However, the dose of green tea extract used independently was four times the dose in the equivalent concentration of NM. Thus, less green tea extract is needed to achieve the same antiviral results when combined with other micronutrients.

Other components in NM include ascorbic acid, amino acids (lysine, proline, arginine), amino-acid derivative (N-acetyl cysteine) and minerals (selenium, copper and zinc), which may also play a role in the suppression of influenza infection. Since the extracellular matrix (ECM) integrity is dependent upon adequate collagen formation and its stability, Rath and Pauling suggested targeting plasmin-mediated mechanisms with the use of nutritional components, such as lysine and lysine analogues [32]. Collagen stability is supported by lysine [32] and also by N-acetyl cysteine NAC) through its inhibitory effect on MMP-9 activity [19] and invasive activities of tumor cells [28]. NAC was shown to significantly decrease mortality in influenza-infected mice [38]. Furthermore, in combination with ribavirin, NAC demonstrated a synergistic effect in protecting mice against a lethal influenza viral infection [10]. In this study, lysine, proline and N-acetyl cysteine demonstrated antiviral activity in avian influenza virus type A/H5N1 infected cells, though not as potent as green tea extract.

Manganese and copper are also essential cofactors in formation of collagen. Ghandi et al. [9] reported an antiviral effect directed towards influenza A virus by copper through inhibition of the proton translocation machinery in the M2 protein of this virus. Selenium has been shown to interfere with MMP expression and tumor invasion [42]. Selenium deficiency has been demonstrated to increase the pathogenicity of influenza virus infection in mice [2].

Finally, in contrast to the toxic side effects of current influenza medications, the nutrient mixture has been shown to be a safe therapeutic agent with demonstrated efficacy. In a previous *in vivo* study addressing safety issues, we found that gavaging adult female ODS rats (weighing 250–300 mg) with the nutrient mixture (at 30, 90 or 150 mg per day for seven days), had neither adverse effects on vital organs (heart, liver and kidney), nor on the associated functional serum enzymes, indicating that this mixture is safe to use even at these high doses, which far exceed the normal equivalent dosage of the nutrient, and can be delivered *in vivo* [36].

In conclusion, the relatively non-toxic nutrient mixture tested in this report demonstrated high antiviral activity as well as therapeutic effects and thus has potential in avian influenza treatment.

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