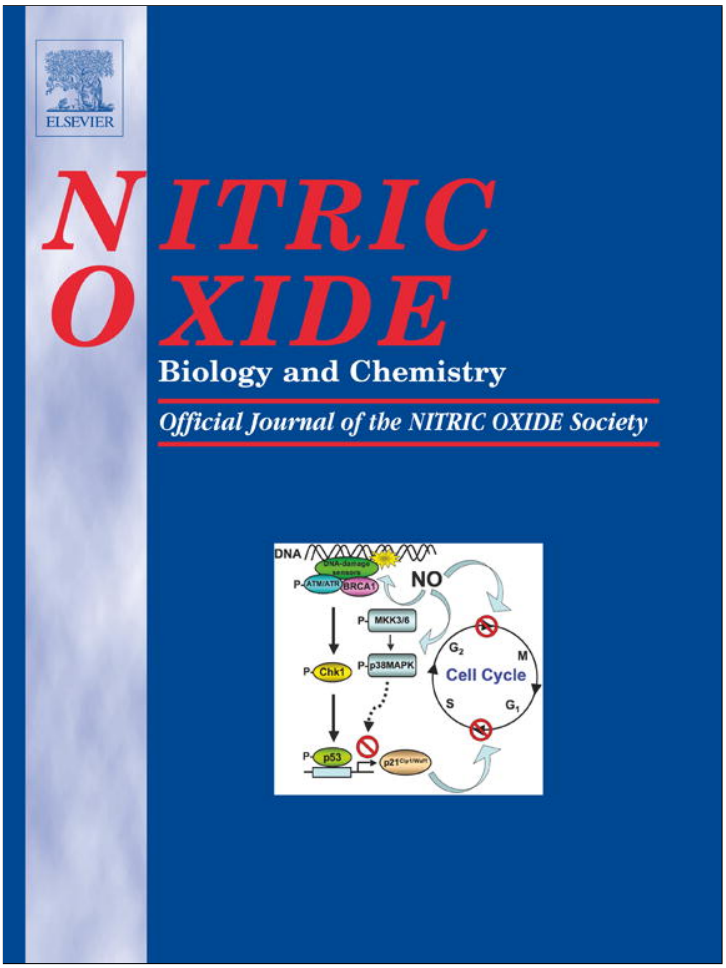

Nitric Oxide 31 (2013) 48–53

Contents lists available at SciVerse ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Gaseous nitric oxide reduces influenza infectivity *in vitro*

Gilly Regev-Shoshani [a,c], Selvarani Vimalanathan [b], Bevin McMullin [a,c,e], Jeremy Road [a], Yossef Av-Gay [c,d], Chris Miller [a,c,*]

[a] Department of Respiratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada
[b] Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada
[c] Division of Infectious Diseases, Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada
[d] Department of Medical Microbiology and Infections Control, Vancouver Coastal Health at Vancouver General Hospital and at University of British Columbia, Vancouver, British Columbia, Canada
[e] Respiratory Services, Vancouver Coastal Health – UBCH Site, Vancouver, British Columbia, Canada

ARTICLE INFO

Article history:
Received 4 July 2012
Revised 22 March 2013
Available online 2 April 2013

Keywords:
Antiviral
Influenza
Nitric oxide
Pandemic
Neuraminidase

ABSTRACT

Gaseous nitric oxide (gNO) is an approved vasodilator drug for inhalation up to a maximum dose of 80 ppm. While gNO has been shown, *in vitro*, to be an effective antibacterial agent (at 160 ppm), NO-donor compounds have been shown to inhibit a variety of viruses at varying stages of replication. This research was done in order to determine whether gNO at 80 or 160 ppm possesses an antiviral effect on influenza viruses. Three strains of influenza (A and B) were exposed to gNO for up to 180 min, before and after infection of MDCK cells. In search for possible mechanism of antiviral action, Neuraminidase (NA) inhibition assay of H1N1 that was exposed to gNO was performed. Results show that when virions were exposed to gNO prior to infection a complete inhibition of infectivity was achieved for all three strains. Post infection exposure of influenza with gNO resulted in about 30% inhibition of infectivity. Further testing showed that when eliminating the pH effect by exposing a dried virus to gNO, 90% inhibition was found after 2 h exposure. NA activity, of whole dried H1N1 virus, was found to be inhibited by gNO (80%). These results suggest that 80 and 160 ppm gNO have a time dependent antiviral effect on influenza strains of viruses during various stages of cellular infection, which are not due to concomitant changes in pH in the surrounding milieu. Viral NA inhibition by gNO was shown and may be responsible for this antiviral effect.

Introduction

For centuries influenza has affected human health both seasonally and with recurring pandemics. Despite significant reduction of disease burden through vaccination efforts, circulation of seasonal influenza A and B viruses cause excess morbidity and mortality, particularly in patients with preexisting pulmonary conditions. It is reported that the seasonal flu is responsible for over 36,000 deaths and 200,000 hospitalizations at a cost of $10 billion each year in the United States [1,2]. Two subtypes of influenza A virus H3N2 and H1N1 have circulated within the human population. Influenza A viruses have a broad host range and thus differ from influenza B that has a limited host range infecting only humans and seals. In these hosts, influenza B viruses can cause significant disease and are a predominant circulating strain of influenza virus in approximately one in every 3 cases [3]. Both influenza A and B viruses present with two types of surface proteins – hemagglutinin (HA) and neuraminidase (NA) and Matrix protein (M2/BM2), which is a proton-selective ion channel protein integral in the viral envelope. Influenza B viruses harbor some interesting genetic differences from influenza A including some additional encoded proteins and may have different characteristics of M2 protein. Specifically, BM2 is translated by a stop/start translation mechanism, which is different from influenza A viruses in which M2 is translated from a spliced transcript [4].

A pandemic can occur when animal viruses acquire mutations directly or by re-assortment with human viruses that adapt them for replication and transmission in human hosts. Recently, the world experienced a global life-threatening phase 6 pandemic caused by a novel swine origin H1N1 virus. During past pandemics, influenza viruses needed more than 6 months to spread allowing sufficient time to develop new vaccines. As experienced, this new H1N1 virus spread worldwide in less than 6 weeks [5]. Had the virulence been higher, the mortality rate prior to the availability of the vaccine would have been catastrophic. The rapid spread of the viral infection recently experienced is of grave concern as the

* Corresponding author. Address: Department of Medicine, Division of Infectious Diseases, University of British Columbia, 2733 Heather St., Vancouver, British Columbia, Canada V5Z-3J5. Fax: +1 604 875 4013.
E-mail address: miller42@mail.ubc.ca (C. Miller).

1089-8603/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.niox.2013.03.007

development window to manufacture an effective vaccine may not be sufficient in order to provide wide-spread global immunization. Thus, viral infection control methods are mainly dependent on antiviral agents.

Two classes of antiviral medications are currently used to treat and prevent influenza infections, the adamantanes and neuraminidase inhibitors. The adamantine derivatives, amantadine and rimantadine, act on the M2 protein of influenza A. They are not effective against influenza B and the development of wide spread amantadine resistance in H3N2 (99%) and H1N1 (10%) strains during 2008–2009 season has limited their utility [2]. Fortunately, this recent pandemic swine origin H1N1 virus and some other influenza A and B viruses are still susceptible to the two NA inhibiting drugs, zanamivir (inhaled) and oseltamivir (oral) [2,6]. Zanamavir (Relenza) and oseltamivir (Tamiflu) are licensed worldwide for treatment and prevention of influenza. Oseltamivir-resistant viruses have recently increased in circulation, especially among the H1N1 virus [7]. New antivirals have been developed in the last few years but given the rapidly evolving nature of antiviral resistance, other options warrant exploration.

Nitric oxide (NO) is a free radical gas molecule that plays a major role in innate immunity, mammalian host defense against infection, modulation of wound healing, vasodilation, neurotransmission and angiogenesis [8,9]. Free NO or NO derived from donating compounds have been reported to exhibit antimicrobial activity during *in vitro* and *in vivo* animal studies [10–13]. The literature supports that NO or its derivatives have inhibitory effects on a variety of viral infections [14]. This inhibitory effect was shown to be marked in IFN–mediated inhibition manifested by activated macrophages [15]. It was also shown to be correlated with s-nitrosylation of viral proteins such as reductases and proteases [reviewed in 16]. Conversely, it appears that despite the seemingly beneficial role of NO in viral infections, the over production of NO in response to viral insult may lead to detrimental effects in the host, particularly in influenza infections [17].

The antiviral effect of NO was previously shown using various NO donor compounds. It has been reported to inhibit replication in both DNA and RNA viruses such as HSV-1, Coxsackie virus, Coronavirus and Dengue virus [18–22]. Rimmelzwaan et al., have shown that replication of influenza A viruses in MDCK cells were severely impaired by the NO donor S-nitro-*N*-acetylpenicillamine (SNAP). They showed that the antiviral effect correlated with inhibition of viral RNA synthesis, indicating that NO may interfere with the early stages of replication [10]. Still, the mechanism of influenza inhibition by NO is not completely understood.

The gaseous form of NO (gNO) has been approved as an inhaled drug for the therapeutic treatment of pulmonary hypertension of the newborn at a concentration of up to 80 parts per million (ppm). It has been shown that gNO doses lower than 80 ppm are not antibacterial [23] while we have shown that an effective antibacterial concentration of gNO is 160 ppm [24,25]. We purport that inhaled gNO may be a useful antimicrobial treatment for pulmonary infections. To our knowledge, there are no reports on the evaluation of the highest approved level (80 ppm) or the higher antibacterial dose of 160 gNO to identify the antiviral potential of NO on influenza viruses.

In this study, we evaluate the effect of gNO on three representative influenza viruses in both an infected cell and cell-free *in vitro* models utilizing our previously validated gNO exposure system [26]. We chose to use a H3N2 strain to represent seasonal influenza, a H1N1 subtype influenza, and an influenza B virus. In order to elucidate a potential anti-viral mechanism of action, we evaluated the effect of gNO on the inhibition of the surface protein NA on the H1N1 virus.

Experimental procedures

Viruses and cell lines

Madin-Darby Canine Kidney Epithelial (MDCK) cells (ATCC CCL-34) were obtained from the American Type Culture Collection and maintained in Dulbecco minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and incubated at 37 °C in a humidified atmosphere with 5% CO_2 without antibiotics or antimycotic agents. MDCK cells were grown as monolayers in 75-cm^2 cell culture flasks. Passages between 3 and 15 were used for these experiments.

All viral strains were obtained from the laboratory stock from the British Columbia Center for Disease Control. Stocks of influenza A viruses, A/Denver/1/1957 (H1N1), A/Victoria/3/75(H3N2) and influenza B Virus, B/Hong Kong/5/72 were grown in MDCK for 48 h, with medium containing 2 μg/ml modified trypsin (treated with TPCK) without serum. All the stock viruses were prepared as clarified cell-free supernatants. Virus concentration for stocks were determined by standard plaque assay on MDCK cells [27]. Virus titers for these stocks were 3×10^7 (H3N2), 6×10^6 (H1N1) and 1×10^5 (influenza B) plaque forming units (PFU)/ml respectively.

Gaseous nitric oxide delivery

The design and validity of the continuous horizontal-flow gNO delivery device used in this study has been described in detail elsewhere [26]. In brief, the device consisted of two cylindrical Plexiglas® exposure chambers with separate gas entry ports and a common exit port. These chambers were surrounded by an airtight Plexiglas® jacket to create a thermally isolated environment. This jacket enclosed an electrical heater unit controlled by an internal thermostat (Invensys Appliances Control, Carol Stream, Illinois, USA), that provided stable temperatures inside the chamber. Independent lines from each of the two exposure chambers provided samples of the gas mixtures to a $NO/NO_2/O_2$ electrochemical analyzer (AeroNOx, Pulmonox Medical Inc, Tofield, AB, Canada) to detect the exact composition of the gases in the mixture. Gases were supplied from pressurized cylinders at a constant pressure of 50 pounds per square inch. These included 10,000 parts per million (ppm) NO diluted in N_2 (Airgas, Chicago, USA), and medical air (Praxair, Mississauga, ON, Canada). These gases were then mixed together at pre-determined concentrations using a dilution manifold and a digital mass flow meter (TSI Inc., Shoreview, MN, USA). Gas mixtures of 80 or 160 ppm, were delivered to the exposure chamber at a rate of 10.0 L/min at 70–90% relative humidity at temperatures of approximately 28–37 °C (appropriate for each experiment), through two independent humidifiers (MR850, Fisher & Paykel Healthcare, CA, USA). Control chamber contained only air flow at 10 L/min.

Post infection effect of gaseous nitric oxide

Confluent monolayers of MDCK cells in 6-well plates were washed once with phosphate buffered saline (PBS) and then infected with influenza virus at 200 PFU/well. The plates were continuously shaken on a shaker for 45–60 min at 37 °C for virus adsorption. The inoculum was removed and replaced with 1 ml of saline (with 0.5% FBS) per well. Infected plates were treated with either 160 ppm gNO (treatment) or air (control) for 1, 2 and 2.5 h and after each time point, saline was removed and replaced with 2 ml/well overlay medium consisting of 2× DMEM supplemented with 0.5% agarose and 2 μg/ml TPCK–trypsin. After 2 days incubation at 37 °C, the infected cells were fixed with 3% buffered forma-

lin, stained with 0.1% crystal violet and the number of plaques was counted. To insure that NO has no effect on the cells, non-infected cell's viability after NO exposure was confirmed.

Virucidal (cell-free) effect of gaseous nitric oxide

1000 PFU/ml of the indicated virus were treated with 80 or 160 ppm gNO, in saline with 0.5% FBS, for 10–180 min. At the end of each time point, virus infectivity was measured using plaque reduction assay. Confluent MDCK cells were grown in 6-well culture plates and infected with virus from control and treated samples to give 100–250 plaques per well (for optimal visualization). The plates were incubated in 5% CO2 at 37 °C for 1 h. Following 1 h absorption, the virus inoculum was removed and cells were then cultured for 2 days with 2 ml/well overlay medium followed by fixation and staining as described above.

Nitrite and pH content

NO has a short half-life *in vivo* of a few seconds. Therefore, the levels of more stable NO metabolites, nitrite and nitrate, were used for indirect measurement of NO in these experiments. Nitrite concentration at the end of each treatment was measured using Griess reagent [28]. A sample (100 l of saline) was taken from each treatment and control plate (one well) and tested for the nitrite concentration and pH.

Nitric oxide effect on dried H1N1

To eliminate the effect of pH on viral inhibition, another experiment was done, using a dried virus. Aliquots of H1N1 virus −20 l with approximately 10,000 PFUs, diluted in saline (plus 0.5% FBS), were spotted onto a sterile glass slide (25 × 15 mm) and let dry in air inside a biosafety cabinet for about 20 min. Glass slides were treated with a flow of 10 l/m of 160 ppm gNO for 60 and 120 min. Controls were treated with 10 l/m air flow. Samples were reconstituted in 1 ml PBS, and virus infectivity was measured using plaque reduction assay, as outlined above.

Effect of nitrite and pH on cell-free influenza virus

Since nitrite and low pH are found in the treated samples, the individual effect of those was tested here. H1N1 (1000 PFU/ml final concentration) was added to 1 ml of a. Saline, b. Saline with 10 mM nitrite, c. Saline with added citric acid to reduced pH to 4.5. After 30 and 60 min virus infectivity was measured using plaque reduction assay, as explained above.

NA inhibition assay

In order to test a possible mechanism of viral inhibition of NO a NA inhibition assay was performed on H1N1 whole virions. The NA titer was established and a 1:1 dilution (in PBS) from viral stock was chosen for the experiment. Aliquots (25 µl) of H1N1 were spotted onto a sterile glass slide (25 × 15 mm) and let dry in air inside a biosafety cabinet for about 15 min. The virus was dried, and not exposed in saline, since the pH changes during treatment may interfere with the enzymatic assay. Glass slides were treated with a flow of 10 l/m of 160 ppm gNO for 30, 60 and 120 min in the exposure chamber described above. Controls were treated with air under the same conditions. Following gas exposure, samples were reconstituted in 50 µl reaction buffer and NA inhibition was assessed. The chemiluminescent neuraminidase activity inhibition assay was conducted using a commercially available kit, Amplex Red (Invitrogen, Paisley, UK). The experiment was repeated 3 times. Values shown represent mean of triplicate analysis.

Statistical analysis

Data in all the above exposure experiments were expressed as mean value of repetitions with standard deviation (S.D.). Statistical analysis of data obtained in all experiments, were performed using a one-way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test. A value of $p < 0.05$ was considered statistically significant. Data analysis and graphical presentation were done using a commercial statistics package (Graphpad-Prism V 3.0, GraphPad Software Inc., USA).

Results

Post infection effect of gaseous nitric oxide in cells

MDCK cells were infected with virions and then treated with either 160 ppm gNO or air (control) for 1, 2 and 2.5 h. This was done to assess whether gNO would have an effect on viral replication.

Viability of control cells was confirmed. The MDCK cells did adhere to the 6-well plate after infection and could be visualized by staining. H1N1 was found to be the most sensitive to gNO, resulting in about 30% reduction of plaques formed after 2 and 2.5 h of treatment (Fig. 1a). No change from control was observed following 2 h treatment post infection with H3N2, while a 25% inhibition was achieved after 2.5 h (Fig. 1b). Treating influenza B infected cells for up to 2.5 h with 160 ppm gNO, did not show any effect on the virus, compared to control (Fig. 1c).

Virucidal effect of gaseous nitric oxide on cell-free virions

All three viruses were suspended in saline then were exposed to 80 or 160 ppm concentrations of gNO and different exposure times. Controls were treated with air for the same period of time. Virus infectivity was measured using a plaque assay with MDCK cells. gNO was shown to have a time and dose dependent effect on all three viruses. As shown in Fig. 2a, exposing the H1N1 to a continuous dose of 80 ppm NO resulted in 20% reduction in ability to infect after an hour, 50% after 2 h and complete inactivation after 3 h. While using 160 ppm resulted in an increase to 65% viral inactivation after 30 min and complete inactivation after an hour. Repeating the same experiment with H3N2 (Fig. 2b) revealed similar results with a little higher susceptibility to NO. Using 80 ppm, the treatment caused a slight reduction of viral load after 1 h and complete inhibition after 2 h. When using 160 ppm on H3N2, complete inhibition was reached after 30 min treatment.

Influenza B showed a similar pattern to influenza A, although being less susceptible to gNO (Fig. 2c) with 50% reduction after 60 min and 100% inhibition after 2 h (using 160 ppm). When a lower gNO concentration was used, a similar pattern to H1N1 was seen.

Nitrite concentration, pH and their effect on cell-free H1N1 virions

It can be seen (Table 1) that when exposing a solution to gNO, nitrite are produced and the pH is reduced. This appears to correlate with time of exposure and concentration of gNO. The pH was reduced to 3.5–4.6 range after treatment of 3 h with 80 ppm gNO or 2 h with 160 ppm. The longer the exposure time and the greater the gNO concentration, the lower the pH became and the higher amount of nitrite found in the solution. A correlation was found between the amount of nitrite that was measured in exposed solution and the percentage of virus inhibition. In general, the higher the nitrite concentration and the lower the pH, the higher the percentage of viral inhibition was found to be.

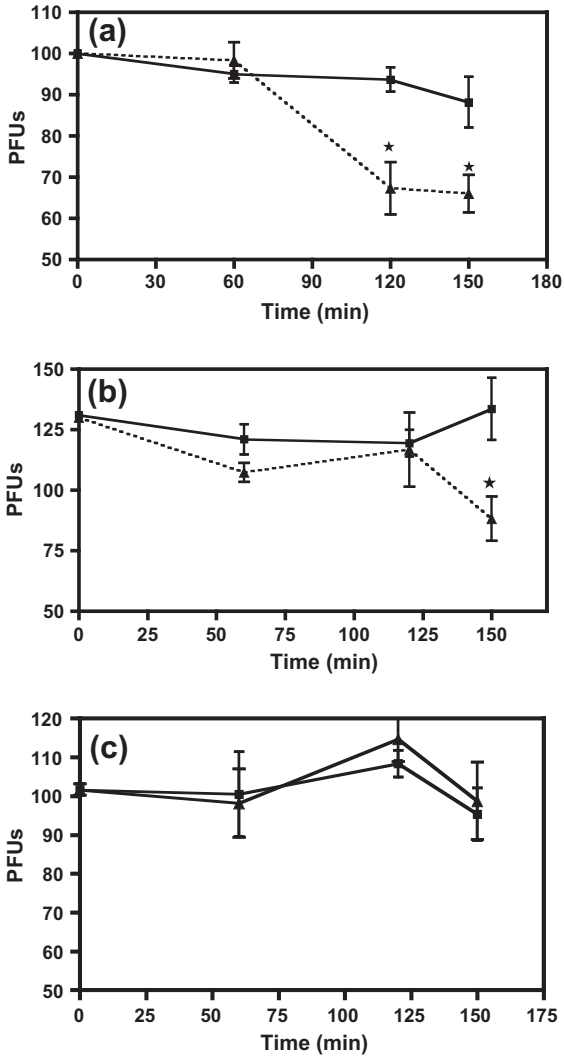

Fig. 1. Post infection inhibition effect of gNO on influenza virus. (a) H1N1, (b) H3N2, (c) Influenza B. Confluent monolayers of MDCK were infected with virus at 100 PFU/well. The plates were continuously shaken on a shaker for 45 min at room temperature for virus adsorption. The solution was removed and replaced with 1 ml of saline per well. Infected plates were treated at 160 ppm gNO for 1, 2 and 2.5 h. At the end of each time point, virus infectivity was measured using plaque reduction assay. Squares represent control (air) while triangles represent gNO exposure. Error bars indicate the standard deviation for two sets of triplicates.

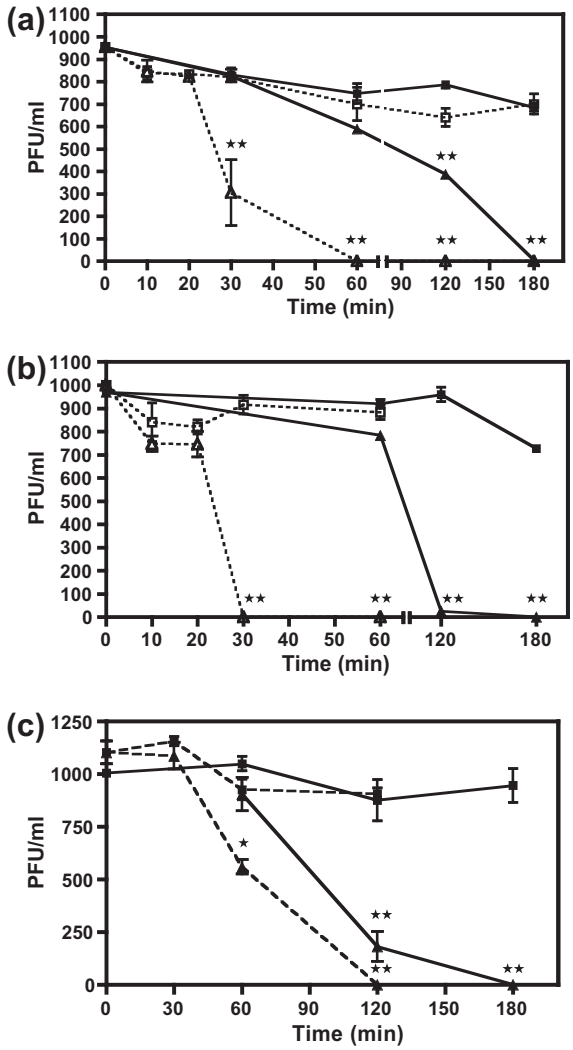

Fig. 2. Virucidal effect of gNO on influenza virus. (a) H1N1, (b) H3N2, (c) Influenza B. Virus (1000 PFUs) in saline was treated with 80 or 160 ppm gNO for 10–120 min. At the end of each time point, virual infectivity was measured using plaque reduction assay. Squares represent control (air) while triangles represent gNO exposure. Straight line used for 80 ppm and dotted line for 160 ppm treatments and controls. Error bars indicate the standard deviation for two sets of triplicates.

To determine whether the pH or the nitrite alone were responsible for the viral inhibition, we tested viral viability after 30 and 60 min in saline, saline with 10 mM nitrite and saline with a reduced pH (4.5). We chose a pH of 4.5 since it was in the range of the pH change we detected after an hour exposure. Both the nitrite alone and the pH of 4.5 alone had no significant effect on the survival of H1N1. Results show that exposing H1N1 to saline with reduced pH (4.5) did not cause reduction in viral load. Following 60 min exposure, there was no significant ($p < 0.001$) change in viral count for pH treatment (850 ± 60) or nitrite treatment (840 ± 75) compared to control (720 ± 68).

In order to eliminate the changes in the surrounding solution (pH) as a factor in the virucidal effect of gNO on the virions, another experiment was done testing the effect of gNO on dried virus

Table 1
Amount of nitrite found in treated and control wells after exposing to NO or air. Nitrite concentration was measured using Griess reagent.

NO concentration (PPM)	Time of exposure (min)	Nitrites (μM)/pH H1N1		Nitrites (μM)/pH H3N2		Nitrites (μM)/pH InfB	
		Tx	Control	Tx	Control	Tx	Control
80	60	156/5.8	23/6.6	174/5.4	21/6.8	193/5.5	5/6.6
80	120	226/5.2	18/6.6	317/4.2	32/6.8	250/4.6	7/6.6
80	180	270/4.6	32/6.5	375/3.5	31/6.7	350/4.1	5/6.6
160	30	171/5.6	13/6.7	198/4.8	15/6.8	133/6.1	8/6.9
160	60	215/5.1	19/6.7	280/4.1	24/6.7	196/5.1	7/6.9
160	120	350/4.1	25/6.7	380/3.7	22/6.7	340/4.4	7.5/6.8

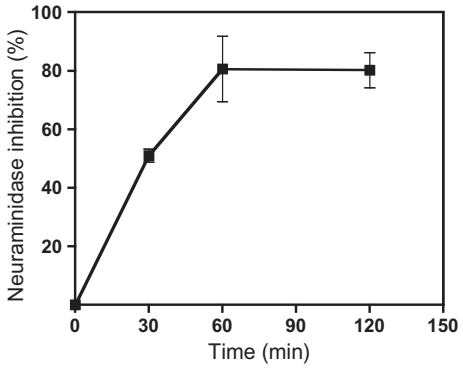

Fig. 3. NA inhibition by gNO. H1N1 was dried on a glass slide and exposed to 160 ppm gNO. Controls were exposed to air. NA inhibition was measured using a commercially available kit and presented as percentage of control following 30, 60, 120 min of gNO exposure.

(H1N1). Previous results [29] indicated that there is about 1 $\log_{10}$ decrease in virus infectivity due to drying. Starting from a titer of 10,000 PFUs, the virus titer was reduced to about 1000 PFUs after drying, before gas exposure. gNO (160 ppm) or air (control) was flowed over the dried virus for 60 and 120 min at 10 lpm. Results show that viral infectivity of the treated virus was reduced by 85% (152 ± 26 PFU for treatment and 980 ± 56 PFU for control) as compared to the control after 2 h of exposure.

NA inhibition assay

NA inhibition of H1N1 by gNO was shown. In order to further clarify the nature of the reaction and to control for the potential pH changes that occur when exposing PBS to gNO we dried the influenza virions onto glass slides before the gas exposures. Control samples were exposed to air while treatment samples were exposed to 160 ppm gNO for 30, 60, 120 min. Following the gas exposure, the dried virions were reconstituted with a reaction buffer and then tested for NA inhibition using a NA Assay kit. Shown in Fig. 3, a 50% inhibition of H1N1 NA was achieved after 30 min of exposure to 160 ppm gNO and 80% inhibition after 60 and 120 min.

Discussion

The results of this study demonstrate that gNO had a significant time and dose dependent effect on the ability of both influenza A and B virions to infect and replicate in MDCK cells. Interestingly, it was shown that virions suspended in normal saline when exposed to gNO lose their ability to infect MDCK cells. Whereas, when MDCK cells were first infected with influenza A, then exposed to gNO, the virucidal effect of gNO was modest but are similar to reported results of an NO donor on influenza [10]. Despite this modest virucidal effect we have previously reported in a complex viral bacteria model in bovine (Bovine Respiratory Disease) that NO reduces the symptoms of the disease. Thus, there may be other downstream host interactions, up regulated by NO, which may not come into play in an *in vitro* study.

The antiviral effect of gNO, in this study, on influenza A during infection is consistent with the effect as shown by Rimmelzwaan et al. using an organic donor, SNAP, on influenza A viruses [10]. They were able to demonstrate that NO released from SNAP inhibits the influenza A virus at an early stage of viral replication which correlated with viral RNA synthesis. Studies, using Coxsackie virus, have demonstrated that NO interrupted the viral life cycle and that this may be due to NO-mediated S-nitrosylation of the cysteine residue inhibiting protease activity in the protease 3C [19,20]. Harris et al. demonstrated that several processes in the late stages of viral replication, including viral DNA replication, viral protein synthesis, and virion maturation, were greatly inhibited by IFN–induced NO in vaccinia virus [30]. Other *in vitro* studies utilizing chemical donors of NO have shown inhibition of viral replication in DNA and RNA viruses. It was suggested that NO inhibits viral proteins, RNA synthesis and viral replication by modifying molecules such as reductases and proteases required for replication [2,18,20,21,31]. To our knowledge, this is the first time that a direct virucidal activity of gNO on cell free virions is reported. There may be targets on the virion, such as HA and NA, to which NO could bind and disrupt the infection process. However, little is known about the antiviral mechanism by which NO acts. One of the plausible mechanisms of antimicrobial activity of NO involves the interaction of this free radical (and a reactive nitrogen intermediate) with reactive oxygen intermediates, such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) to form a variety of antimicrobial molecular species [32]. Colasanti et al. theorized that nitric oxide may be able to affect surface proteins, by nitrosylation of the cysteine moieties within its structure [16]. This could alter the stoichiometry interaction with sialic acid or prevent the fusion of the virion with the epithelial cell membrane [33]. Results from this study seem to support the notion.

The influenza viruses' surface glycoproteins, HA and NA, are the antigens that define the particular strain of influenza. The variation of these molecules over time permits the virus to evade human immune responses and therefore necessitates the formulation of a new vaccine each year. The HA is a sialic acid receptor-binding molecule and mediates entry of the virus into target cells. Whereas, the NA cleaves the cellular-receptor sialic acid residues to which the newly formed particles are attached. This cleavage releases the viruses, which can then invade new cells. Like other NA inhibitors, without functional NA, infection could be limited to one round of replication, rarely enough to cause disease. Preliminary results, shown here for the first time, demonstrate that when testing on a whole virion, NA activity is inhibited by gNO. Thus, NA inhibition may be one possible mechanism of viral inhibition by NO. We are currently screening a wide variety of viruses to gNO to assess which are more or less susceptible to gNO. Concurrently, we are also evaluating NA and HA inhibition in these viruses. Together, this hopefully will shed further light on the antiviral mechanism caused by gNO.

We demonstrate here that there is a correlation between the length of time the saline, (media which suspends the virions) is exposed to gNO and antiviral effectiveness. Regardless of the gNO concentration (80 or 160 ppm) the antiviral effect coincided with a specific range of nitrite concentration and acidic pH that were dependent on the exposure time. This can be explained by the fact that, over time, gNO diffuses into the saline and results in increasing the nitrite concentration. These ions react with protons in the water and produce HNO_2 resulting in a drop in pH. This resulting pH level along with the nitrite concentration is within the same range as was found to be antifungal and antibacterial in other studies using a combination of acid and nitrite (acidified nitrite) producing NO gas [34–36]. Although low pH levels on their own, can have an antiviral effect, we show here that by eliminating the acidified liquid milieu we still achieve a reduced cellular infectivity of H1N1. The gNO treatment in this case showed reduced infectivity (by 85%) and thus provides further evidence that there are targets on the virion that NO may bind with and prevent cellular infectivity. This notion was further supported by the results from the NA assay. Moreover, We have shown here that when adding virus into either 10 mM nitrite at a neutral pH or to saline with a reduced pH (4.5), for 1 h, no effect on virus viability was found. This confirms our conclusions, which support the thought that the NO is the antiviral compound in this reaction.

In this study, gNO was shown to inhibit NA activity of H1N1. Nitrosylation of surface proteins may be the mechanism of inhibition as it may change surface protein structure (like HA and NA) on the virions and thus cause reduced infectivity. This should be further investigated.

We conclude that gNO has an antiviral effect on the influenza H3N2 (seasonal flu), H1N1 (pandemic flu) and influenza B. This effect is dose dependent and begins to occur at the highest range of dosages applied in the approved use of inhaled NO for full term infants (80 ppm). At a dose of 160 ppm a significant virucidal and an antiviral effect during early infection of influenza A was observed. However influenza B was not similarly affected during infection but was susceptible in a cell free environment. We propose here a mechanism of action for the viral inhibition, in which influenza NA is being inactivated by gNO. Future research should focus on expanding these experimental observations to test the antiviral effect of gNO and surface protein (HA and NA) inhibition on a wide variety of viruses in order to help elucidate the mechanism of its antiviral action.

Acknowledgments

Financial support for this study was provided by Grants from Lotte & John Hecht Memorial Foundation (YA, CM), The US Defense Advanced Research Projects Agency F10-02374 (CM,YA) and Nitric Solutions Inc. (GR). CM is the founder of Nitric Solutions Inc. We would like to thank the British Columbia Centre for Disease Control for providing viral strains.

References

[1] W.W. Thompson, D.K. Shay, E. Weintraub, L. Brammer, N. Cox, L.J. Anderson, K. Fukuda, Mortality associated with influenza and respiratory syncytial virus in the United States, JAMA 289 (2003) 179–186.
[2] P.K. Tosh, R.M. Jacobson, G.A. Poland, Influenza vaccines: from surveillance through production to protection, Mayo Clin. Proc. 85 (2010) 257–273.
[3] CDC. Update: Influenza Activity – United States and Worldwide, May 22–September 3, MMWR 60 (2011) 1239–1242.
[4] P. Palese, M.L. Shaw, Orthomyxoviridae: the viruses and their replication, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, Lippincott Williams & Wilkins, Philadelphia, 2007, pp. 1647–1689.
[5] http://www.who.int/csr/don/2009_07_27/en/index.html.
[6] A. Moscona, Neuraminidase inhibitors for influenza, N. Engl. J. Med. 353 (2005) 1363–1373.
[7] N.J. Dharan, L.V. Gubareva, J.J. Meyer, M. Okomo-Adhiambo, R.C. McClinton, S.A. Marshall, K. St George, S. Epperson, L. Brammer, A.I. Klimov, J.S. Bresee, A.M. Fry, Infections With Oseltamivir-Resistant Influenza A(H1N1) Virus in the United States, JAMA 301 (2009) 1034–1041.
[8] F.Y. Liew, F.E. Cox, Nonspecific defence mechanism: the role of nitric oxide, Immunol. Today 12 (1991) A17–A21.
[9] M. Rizk, M.B. Witte, A. Barbul, Nitric oxide and wound healing, World J. Surg. 28 (2004) 301–306.
[10] G.F. Rimmelzwann, M.M. Baars, P. Lijster, R.A. Fouchier, A.D. Osterhaus, Inhibition of Influenza virus replication by nitric oxide, J. Virol. 73 (1999) 8880–8883.
[11] R. Weller, R.J. Price, A.D. Ormerod, N. Benjamin, C. Leifert, Antimicrobial effect of acidified nitrite on dermatophyte fungi, Candida and bacterial skin pathogens, J. Appl. Microbiol. 90 (2001) 648–652.
[12] A. Ghaffari, C.C. Miller, B. McMullin, A. Ghahary, Potential application of gaseous nitric oxide as a topical antimicrobial agent, Nitric Oxide 14 (2006) 21–29.
[13] G. Regev-Shoshani, M. Ko, C. Miller, Y. Av-Gay, Slow release of nitric oxide from charged catheters and its effect on biofilm formation by *Escherichia coli*, Antimicrob. Agents Chemother. 54 (2010) 273–279.
[14] C.S. Reiss, T. Komatsu, Does nitric oxide play a critical role in viral infections?, J Virol. 72 (1998) 4547–4551.
[15] R. Koetzler, R.S. Zaheer, R. Newton, D. Proud, Nitric oxide inhibits IFN regulatory factor 1 and nuclear factor kB pathways in rhinovirus-infected epithelial cells, J. Allergy Clin. Immunol. 124 (2009) 551–557.
[16] M. Colasanti, T. Persichini, G. Venturini, P. Ascenzi, S-Nitrosylation of viral proteins: molecular bases for antiviral effect of nitric oxide, IUBMB Life 48 (1999) 25–31.
[17] T. Akaike, H. Maeda, Nitric oxide and virus infection, Immunology 101 (2000) 300–308.
[18] K.D. Croen, Evidence of nitric oxide. Inhibition of herpes simplex virus type 1 replication, J. Clin. Invest. 91 (1993) 2446–2452.
[19] M. Saura, C. Zaragoza, A. McMillan, R.A. Quick, C. Hohenadi, J.M. Lowenstein, An antiviral mechanism of nitric oxide: inhibition of viral protease, Immunity 10 (1999) 21–28.
[20] R. Zell, R. Markgraf, M. Schmidtke, A. Stelzner, A. Henke, H.H. Sigusch, B. Glück, Nitric oxide donors inhibit the coxsackievirus B3 proteinases 2A and 3C *in vitro,* virus production in cells, and signs of myocarditis in virus-infected mice, Med. Microbiol. Immunol. 193 (2004) 91–100.
[21] S. Akerström, M. Mousavi-Jazi, J. Klingström, M. Leijon, A. Lundkvist, A. Mirazimi, Nitric oxide inhibits the replication cycle of severe acute respiratory syndrome coronavirus, J. Virol. 79 (2005) 1966–1969.
[22] W. Charnsilpa, R. Takhampunya, T.P. Endy, M.P. Mammen, D.H. Libraty, S. Ubol, Nitric oxide radical suppresses replication of wild-type dengue 2 viruses *in vitro*, J. Med. Virol. 77 (2005) 89–95.
[23] R. Long, J. Talbot, R.L. Mayers, R.L. Jones, Treatment of sputum-smear positive pulmonary tuberculosis with inhaled nitric oxide, Antimicrob. Agents Chemother. 49 (2005) 1209–1212.
[24] C. Miller, B. McMullin, A. Ghaffari, A. Stenzler, N. Pick, D. Roscoe, A. Ghahary, J. Road, Y. Av-Gay, Gaseous nitric oxide bactericidal activity retained during intermittent high-dose short duration exposure, Nitric Oxide 20 (2009) 16–23.
[25] B. McMullin, D. Chittock, D. Roscoe, H. Garcha, L. Wang, C. Miller, The antimicrobial effect of nitric oxide on the bacteria that cause nosocomial pneumonia in mechanically ventilated patients in the ICU, Respir. Care 50 (2005) 1451–1456.
[26] A. Ghaffari, D.H. Neil, A. Ardakani, J. Road, A. Ghahary, C.C. Miller, A direct nitric oxide gas delivery system for bacterial and mammalian cell cultures, Nitric Oxide 12 (2005) 129–140.
[27] F.G. Hayden, K.M. Cote, R.G. Douglas, Plaque inhibition assay for drug susceptibility testing of influenza viruses, Antimicrob. Agents Chemother. 17 (1980) 865–870.
[28] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, S.R. Tannenbaum, Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids, Anal. Biochem. 126 (1982) 131–138.
[29] J.B. Hudson, M. Sharma, S. Vimalanathan, Development of a practical method for using ozone gas as a virus decontaminating agent, Ozone Sci. Eng. 31 (2009) 216–223.
[30] N. Harris, R.M. Buller, G. Karupiah, Gamma interferon-induced, nitric oxide-mediated inhibition of vaccinia virus replication, J. Virol. 69 (1995) 910–915.
[31] C. Zaragoza, C.J. Ocampo, M. Saura, A. McMillan, C.J. Lowenstein, Nitric oxide inhibition of coxsackievirus replication *in vitro*, J. Clin. Invest. 100 (1997) 1760–1767.
[32] R. Radi, J.S. Beckman, K.M. Bush, B.A. Freeman, Peroxynitrite oxidation of sulfhydryls: the cytotoxic potential of superoxide and nitric oxide, J. Biol. Chem. 266 (1991) 4244–4250.
[33] C. Fischer, B. Schroth-Diez, A. Herrmann, W. Garten, H.D. Klenk, Acylation of the influenza hemagglutinin modulates fusion activity, Virology 248 (1998) 284–294.
[34] R. Weller, R.J. Price, A.D. Ormerod, N. Benjamin, C. Leifert, Antimicrobial effect of acidified nitrite on dermatophyte fungi, Candida and bacterial skin pathogens, J. Appl. Microbiol. 90 (2001) 648–652.
[35] M. Anyim, N. Benjamin, M. Wilks, Acidified nitrite as a potential antifungal agent, Int. J. Antimicrob. Agents 26 (2005) 85–87.
[36] B.J. Hardwick, A.T. Tucker, M. Wilks, A. Johnston, N. Benjamin, A novel method for the delivery of nitric oxide therapy to the skin of human subjects using a semi-permeable membrane, Clin. Sci. 100 (2001) 395–400.