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# Preparation of Nitric Oxide Releasing Solution and Assessment of in Vitro Efficacy on Sars-Cov-2 Virus Using Plaque Assay

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**ABSTRACT**

In our present research, a method for preparing acidified nitrite solution comprising of biocompatible liquids which is able to release an active ingredient Nitric oxide (NO) gas at a pH scope of 3-4 is revealed. By performing an In-Vitro viral plaque assays, the cytopathic effect of NO on respiratory virus such as SARS-CoV-2 was determined. We discovered that the number of plaques reduced in a dose dependent manner as viral entities were found sensitive towards NO gas. Hence, we assert that our NO releasing acidified nitrite solution is relevant as it has shown a cytopathic effect and inhibited the replication of respiratory viruses like SARS-CoV-2 and other rhinovirus spp. Besides, a convenient and portable delivery system has been provided for administration of NO gas instead of cylinder-based system.

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## Introduction

SARS-CoV-2 (Severe acute respiratory syndrome coronavirus-2) arose in 2019 and rapidly spreaded over the world. As of November 2022, over 636 million cases and 6.6 million deaths have been reported worldwide, including more than 44 million cases and 5.3 lakhs deaths in India. Immunizations have been regulated quickly across the globe, including total of 2.1 billion vaccine doses in India. The mucosal surfaces of the respiratory system serve as the primary site of infection, since the respiratory tract is the SARS-CoV-2 virus's major route of entrance. SARS-CoV-2 infection is triggered by the spike (S) surface glycoprotein, which is the primary target of SARS-CoV-2-neutralizing antibodies. The S protein attaches to certain cell surface receptors; in the case of SARS-CoV, it is the angiotensin-converting enzyme 2 (ACE2), and in order to function, the S protein must go through several post-translational modifications such as glycosylation. Furthermore, it was recently demonstrated that S-palmitoylation of the spike protein's endodomain is important for cell fusion mediation.

Nitric oxide (NO), a free radical, available in the form of gas plays an important role in innate immunity, host defence mechanism, vasodilation, neurotransmitter and in antimicrobial activity. Studies revealed that NO has been shown to have non-specific antiviral effects in a range of viral infections, including HIV, influenza, wild type dengue virus, vaccinia virus, enterovirus, and coronavirus. NO or its derivatives reduces the replication of SARS-CoV at early stage and reduce the palmitoylation of the S protein, which is important for S-mediated cell–cell fusion, thereby reducing the attachment of S protein on the angiotensin-converting enzyme 2 (ACE 2) receptor. NO is mainly produced endogenously in endothelial cells of paranasal sinus and nasal mucosa from L-arginine and L-citrulline by a family of NO synthase enzymes (NOS). NO regulates the ciliary beat frequency, when working perfectly, creating a sterile environment in the nasal cavity.

Nitric oxide (NO) is an approved pulmonary vasodilator that is recommended for neonates and full-term babies up to a dosage of 80 ppm. Above 80 ppm, NO can lead to toxic side effects by binding to haemoglobin results in methemoglobin, which may reduce oxygen transport and NO's ability to function as a nitrosylating agent on proteins and other cell components. However, clinical studies have shown that delivery of 160 ppm NO 5 times a day for 5 days is safe for humans. This study aims on the development of acidified nitrite solution which is able to release NO in ppm concentrations consistently for long period of time which can be stored in a portable device to use it anytime and anywhere. Further, the in-vitro effect of NO on cytopathic activity of SARS-CoV-2 virus was evaluated using Vero E6 cell lines.

## Materials and Methods

### Preparation of No Releasing Acidified Nitrite Solution (Norans)

All process was carried out in a clean room environment to reduce the chances of contamination and glassware was sterilized using autoclave.Preparation of 1.2% w/v Sodium nitrite solution (solution A).

1.2g Sodium nitrite USP grade was accurately weight using Mettler Toledo Excellence Plus, Model No: XP205 weighing machine. The weighed sodium nitrite was mixed in 50 ml 0.9% saline solution and mixed properly using magnetic stirrer. The pH of the solution was measured and recorded.

Preparation of 30% w/v citric acid (pH adjuster) solution (solution B):6g Citric acid was accurately weight using Mettler Toledo Excellence Plus, Model No: XP205 weighing machine. The weighed citric acid was mixed in 8 ml 0.9% saline solution and mixed properly using magnetic stirrer. More saline was added after complete dissolution, to make up the solution to 10 ml. The pH of the solution was measured and recorded.

Acidification of solution A: Solution A was acidified by adding Solution B drop wise until the pH reached to 3.4. The resultant solution was made up to 100 ml using saline and pH was adjusted to 3.4, if necessary.

**Storage of No Releasing Releasing Acidified Nitrite Solution (Norans)**

The NORANS was stored in a plastic bottle and screw capped with 100 microlitre spray pump and actuator as seen in Figure 1. Similarly, the solution was packed in a plastic bottle containing 200 microlitre, 300 microlitre, 400 microlitre and 500 microlitre spray pumps.

**Figure 1:** Plastic Bottle Containing NORANS for Storage and Delivery of Solution

**Measurement of Nitric Oxide Gas Release from NORANS**

The NO release form NORANS was measured using portable NO gas analyzer purchased from Endee Engineers Pvt. Ltd which can detect up to maximum of 500 parts per million (ppm) of NO.

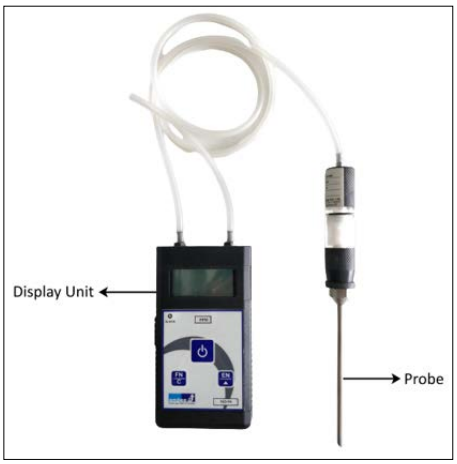


**Figure 2:** Portable NO Gas Analyser

The portable NO gas analyzer as shown in Figure 2, consists of a probe, a connector tube and an analyzer. The probe consists of long stainless-steel rod which is used for suction of NO gas which is transferred to the analyzer using the connector tube. The analyzer contains the sensor which detects the NO gas and display the results in PPM level on the screen. To measure the NO release from the prepared NORANS, single spray of 100 µL solutions was administered in a glass bottle and screw caped. The solution was allowed to settle down for 60 seconds and then uncapping the bottle, the probe of portable NO gas analyzer was inserted in the bottle and the NO gas was measured using analyzer. Similarly, single spray of 200 µL, 300 µL, 400 and 500 µL of NORANS was administered in the bottle. Likewise, double spray of 100 µL, 200 µL, 300 µL, 400 and 500 µL of NORANS was administered in the bottle.

**In-Vitro Efficacy of Norans on Sars-Cov-2 Virus Using Plaque Assay**

To investigate the role of NO in SARS-CoV-2 infection, we infected Vero E6 cells with SARS-CoV-2 positive sample in Viral Transport Media (VTM). Vero E6 cells are highly identical and susceptible to lung cells for SARS-CoV-2 infections and virus propagation in these cells is much more efficient. By testing different overlays and assay formats we developed cytopathogenic plaque assays that can be used for analytical and preparative purposes. Plaque assays make use of viscous overlays to cover cells immediately after infection, thus limiting virus spread and restricting virus growth to foci of cells at the sites of initial infection. If virus contributes no or low cytopathic effects to cells, these foci may be visualized by immune staining. If virus induces strong cytopathogenic effects (CPE), cells in plaques are lysed and plaques can be visualized by staining of the residual intact cells. Cytopathogenic plaque assays are compatible with high throughput screening, it is helpful in obtaining virus stocks of optimized infectivity.SARS-CoV-2 Positive VTM sample was procured from Shree Vinoba Bhave Civil Hospital, Silvassa and number of virus copy was estimated to 20000 copies/mL using polymerase chain reaction (PCR) machine. Vero E6 cells were procured from ECACC General Cell Collection and activated by transferring to Complete IMDM media (Filter sterilized 10 mL of Iscove's Modified Dulbecco's Medium (IMDM) with 10% Fetus bovine serum (FBS) & 1% Glutamine) in sterilized condition, incubated in incubator at 37 °C for 72 h. As stated in table 1, a mixture of 900µL IMDM + 100 µL Vero E6 cells + 10 µL FBS + 1 µL Glutamine served as negative control and the same mixture along with 200 µL viral VTM served as positive control. Negative

control along with test sample of single dose - different volume of NORANS i.e. 100 µL, 200 µL, 300 µL, 400 µL and 500 µL were applied on the sterile petri plates and was allowed to dry in sterile condition. Similarly, negative control along with test sample of double dose - different volume of NORANS i.e. 100 + 100 µL, 200 + 200 µL, 300 + 300µL, 400 + 400µL and 500 + 500µL were applied on the sterile petri plates and was allowed to dry in sterile condition. In double dose, another dose was given after 4 hours after giving first dose. After drying all the plates were overlayered with 1.5% of sterilized agarose and solidified, incubated at 37 °C for 4 days. After incubation 0.1% crystal violet solution were flooded for plaque observation.

**Table 1: Plaque Assay Experimental Design**

| Sr. no. | Test Sample | Experiment design | NORANS (µL) |
|---|---|---|---|
| 1 | Negative control | 900µL IMDM + 100 µL Vero E6 cells + 10 µL FBS + 1 µL Glutamine | 00 |
| 2 | Positive control | (900µL IMDM + 100 µL Vero E6 cells + 10 µL FBS + 1 µL Glutamine + 200 µL viral VTM) | 00 |
| **Single dose of NORANS** | | | |
| 3 | 100 | (900µL IMDM + 100 µL Vero E6 cells + 10 µL FBS + 1 µL Glutamine + 200 µL viral VTM) | 100 |
| 4 | 200 | | 200 |
| 5 | 300 | | 300 |
| 6 | 400 | | 400 |
| 7 | 500 | | 500 |
| **Double dose of NORANS** | | | |
| 8 | 100 + 100 | (900µL IMDM + 100 µL Vero E6 cells + 10 µL FBS + 1 µL Glutamine + 200 µL viral VTM) | 100 + 100 |
| 9 | 200 + 200 | | 200 + 200 |
| 10 | 300 + 300 | | 300 + 300 |
| 11 | 400 + 400 | | 400 + 400 |
| 12 | 500 + 500 | | 500 + 500 |

## Results and Discussion

### Measurement of Nitric Oxide Gas Release from NORANS

NORANS was prepared using 1.2% sodium nitrite solution acidifies to 3.4 pH using 30%w/v citric acid. To measure the NO gas release from prepared NORANS, single spray of 100 µL, 200 µL, 300 µL, 400 and 500 µL and double spray of 100 µL, 200 µL, 300 µL, 400 and 500 µL of NORANS was administered in a bottle. Using the portable NO gas analyzer, the NO release was measured and interpreted in Figure 3.

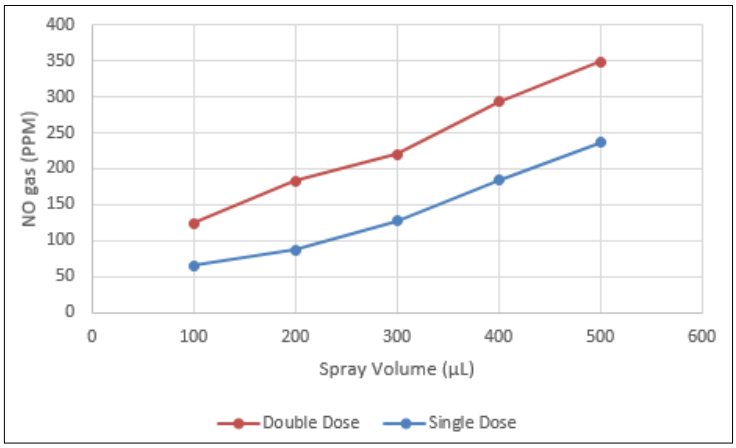


**Figure 3:** Release of NO gas from different spray volume of single and double dose

A single spray of 100 µL was able to release around 65 ± 10 ppm NO gas, while 200 µL spray was able to release around 87 ± 10 ppm NO gas, 300 µL spray was able to release around 128 ± 10 ppm NO gas, 400 µL spray was able to release around 184 ± 10 ppm NO gas and 500 µL spray was able to release around 236 ± 10 ppm NO gas. A double spray of 100 µL was able to release around 124 ± 10 ppm NO gas, while 200 µL spray was able to release around 183 ± 10 ppm NO gas, 300 µL spray was able to release around 220 ± 10 ppm NO gas, 400 µL spray was able to release around 293 ± 10 ppm NO gas and 500 µL spray was able to release around 349 ± 10 ppm NO gas.

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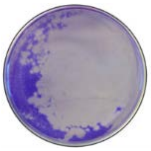
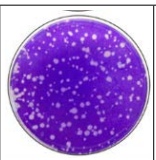
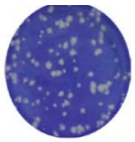
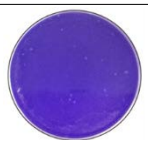
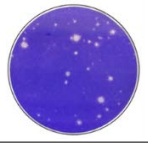
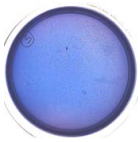
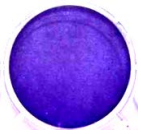
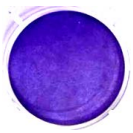
**In-Vitro Efficacy of Norans on Sars-Cov-2 Virus Using Plaque Assay**

To investigate the cytopathic effect (CPE) of NO, we infected vero E6 cells (procured from ECACC General Cell Collection) with SARS-CoV-2 positive VTM samples (procured from Shree Vinoba Bhave Civil Hospital, Silvassa) as plaque-forming units. Single dose and double dose of 100 µL, 200 µL, 300 µL, 400 and 500 µL each respectively was taken as test samples. 5 days later plates were stained with 0.1% crystal violet and observed as below table. As concentration and doses of liquid nasal spray were increased, CPE decreases against SARS-CoV-2 that is observed in tests.

**Table 2: Plaque Assay Observation**

| Sr. No. | Test Sample | Observation | Number of plaques | % Reduction in plaques |
|---|---|---|---|---|
| 1 | Negative Control |  | No plaques | 00 |
| 2 | Positive Control |  | Merged Uncountable Plaques observed | Uncountable Max 300 plaques considered as 100% |
| **Single dose of NORANS** | | | | |
| 3 | 100 |  | 152 | 50 |
| 4 | 200 |  | 136 | 55 |
| 5 | 300 |  | 56 | 81.34 |
| 6 | 400 |  | 6 | 98 |
| 7 | 500 |  | 0 | 100 |
| **Double dose of NORANS** | | | | |
| 8 | 100 + 100 |  | 35 | 88 |
| 9 | 200 + 200 |  | 20 | 93 |
| 10 | 300 + 300 |  | 0 | 100 |
| 11 | 400 + 400 |  | 0 | 100 |
| 12 | 500 + 500 |  | 0 | 100 |

According to results and observation given in table 2, single dose of 400 µL NORANS is optimal to reduce 98% of viral reduction as cytopathic effect observed in terms of plaque. Where as in case of double dose of 200 µL 4 hours intervals is effective in reducing 93% viral reduction while double dose of 300 µL with 4 hours intervals is effective in reducing 100% viral reduction as per plaque reduction observed.

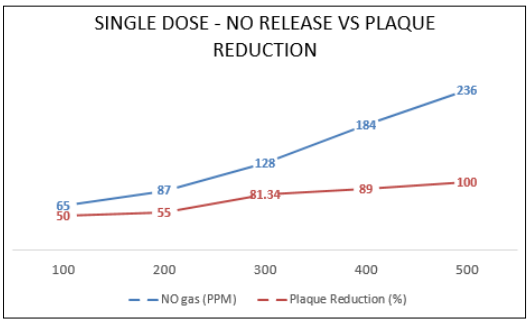



**Figure 4:**% Reduction of Plaques from NO gas Release from Single Dose of NORANS

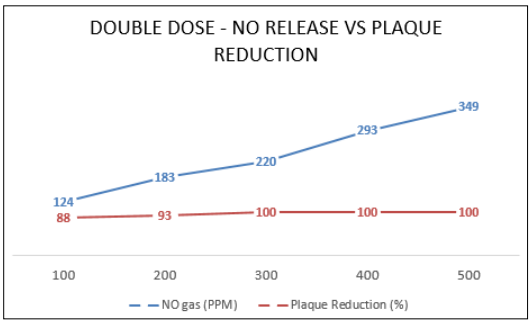



**Figure 5:** % Reduction of Plaques from NO Gas Release from Double Dose of NORANS

As per Figure 4, in a single dose study plaque assay, 65 ppm NO gas, 87 ppm, 128 ppm, 184 ppm and 236 ppm NO gas was able to reduce 50% plaque, 55% plaque, 81.34% plaque, 89% plaque and 100% plaque respectively. While as per data provided in Fig. 5, in double dose plaque assay, 124 ppm, 183 ppm and 220 ppm NO

gas was able to reduce 88%, 93% and 100% plaque respectively.

## Conclusion

An acidified nitrite solution was prepared using 1.2% w/v sodium nitrite and acidified to pH 3.4 using 30% w/v citric showed NO gas release and dose dependent antiviral effect on SARS-CoV-2 virus grown on Vero E6 cell lines. The NORANS solution was stored in a plastic bottle as shown in Figure 1, useful for administration of NORANS and could be a possible portable alternative for NO gas delivery compared to cylinder based NO gas delivery which can be used in healthcare settings only. Data represented here shows that single dose of 400 µl of NORANS is able to produce 184 ± 10 ppm NO gas which reduced 89% plaque compared to positive control. While 500 µl of NORANS was able to produce 236 ppm NO gas which reduced 100% plaque compared to positive control. Whereas, double dose of 200 µl of NORANS which was given at an interval of 4 hours was able to produce 183 ± 10 ppm NO gas which reduced 93% plaque compared to positive control. While 300 µl of NORANS was able to produce 220 ppm NO gas which reduced 100% plaque compared to positive control.

The reduction in plaque by antiviral viral effect of NO could be by two mechanisms as stated by [1]. Firstly, the NO could cause a reduction in viral RNA production in the early steps of viral replication and/or secondly NO could cause a reduction in the palmitoylation of nascently expressed spike (S) protein, which affects the fusion between the S protein and its receptor, angiotensin converting enzyme 2. Further clinical research is required to evaluate the potential effect of high dose (> 80 ppm) in patients with SARS-CoV-2 infections [2-29].

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