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TITLE:

A Novel Inhalation Mask System to Deliver High Concentrations of Nitric Oxide Gas in Spontaneously Breathing Subjects

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KEYWORDS:

Nitric Oxide; Critical Care; Pulmonary Disease; Medical Gas; COVID-19; SARS-CoV-2; Viral Pneumonia; Infectious Disease Transmission; Healthcare Workers

SUMMARY:

This simple and highly adaptable system device for the inhalation of high-concentration nitric oxide (NO) gas does not require mechanical ventilators, positive pressure, or high gas flows. Standard medical consumables and a snug-fitting mask are used to safely deliver NO gas to spontaneously breathing subjects.

ABSTRACT:

Nitric Oxide (NO) is administered as gas for inhalation to induce selective pulmonary vasodilation. It is a safe therapy, with few potential risks even if administered at high concentration. Inhaled NO gas is routinely used to increase systemic oxygenation in different disease conditions. The

administration of high concentrations of NO also exerts a virucidal effect in vitro. Owing to its favorable pharmacodynamic and safety profiles, the familiarity in its use by critical care providers, and the potential for a direct virucidal effect, NO is clinically used in patients with coronavirus disease-2019 (COVID-19). Nevertheless, no device is currently available to easily administer inhaled NO at concentrations higher than 80 parts per million (ppm) at various inspired oxygen fractions, without the need for dedicated, heavy, and costly equipment. The development of a reliable, safe, inexpensive, lightweight, and ventilator-free solution is crucial, particularly for the early treatment of non-intubated patients outside of the intensive care unit (ICU) and in a limited-resource scenario. To overcome such a barrier, a simple system for the non-invasive NO gas administration up to 250 ppm was developed using standard consumables and a scavenging chamber. The method has been proven safe and reliable in delivering a specified NO concentration while limiting nitrogen dioxide levels. This paper aims to provide clinicians and researchers with the necessary information on how to assemble or adapt such a system for research purposes or clinical use in COVID-19 or other diseases in which NO administration might be beneficial.

INTRODUCTION:

NO inhalation therapy is regularly used as a life-saving treatment in several clinical settings[1–3]. In addition to its well-known pulmonary vasodilator effect[4], NO displays a broad antimicrobial effect against bacteria[5], viruses[6], and fungi[7], particularly if administered at high concentrations (>100 ppm).[8] During the 2003 Severe Acute Respiratory Syndrome (SARS) outbreak, NO showed potent antiviral activity in vitro and demonstrated therapeutic efficacy in patients infected with the SARS-Coronavirus (SARS-CoV)[9,10]. The 2003 strain is structurally similar to SARS-Cov-2, the pathogen responsible for the current Coronavirus Disease-2019 (COVID-19) pandemic[11]. Three randomized controlled clinical trials are ongoing in patients with COVID-19 to determine the potential benefits of breathing high-concentration NO gas to improve outcomes[12–14]. In a fourth ongoing study, the prophylactic inhalation of high concentrations of NO is being investigated as a preventive measure against the development of COVID-19 in healthcare providers exposed to SARS-CoV-2-positive patients[15].

The development of an effective and safe treatment for COVID-19 is a priority for the healthcare and scientific communities. To investigate the administration of NO gas at doses > 80 ppm in non-intubated patients and volunteering healthcare workers, the need to develop a safe and reliable non-invasive system became apparent. This technique aims to administer high NO concentrations at different fractions of inspired oxygen (FiO_2) to spontaneously breathing subjects. The methodology described here is currently in use for research purposes in spontaneously breathing COVID-19 patients at the Massachusetts General Hospital (MGH)[16,17]. Following the guidelines of MGH's human research ethics committee, the proposed system is currently in use to conduct a series of randomized controlled trials to study the following effects of high concentrations of NO gas. First, the effect of 160 ppm NO gas is being studied in non-intubated subjects with mild-moderate COVID-19, admitted either in the Emergency Department (IRB Protocol #2020P001036)[14] or as inpatients (IRB Protocol #2020P000786)[18]. Second, the role

of high-dose NO is being examined to prevent SARS-CoV-2 infection and the development of COVID-19 symptoms in healthcare providers routinely exposed to SARS-CoV-2-positive patients (IRB Protocol # 2020P000831)[19].

This simple device can be assembled with standard consumables routinely used for respiratory therapy. The proposed apparatus is designed to non-invasively deliver a mixture of NO gas, medical air, and oxygen (O_2). Nitrogen dioxide (NO_2) inhalation is minimized to reduce the risk of airway toxicity. The current NO_2 safety threshold set by the American Conference of Governmental Industrial Hygienists is 3 ppm over an 8-h time-weighted average, and 5 ppm is the short-term exposure limit. Conversely, the National Institute for Occupational Safety and Health recommends 1 ppm as a short-term limit of exposure[20]. Given the increasing interest in high-dose NO gas therapy, the present report provides the necessary description of this novel device. It explains how to assemble its components to deliver a high concentration of NO for research purposes.

PROTOCOL:

NOTE: See the **Table of Materials** for the materials needed to assemble the delivery system. Sources of medical air, O_2, and NO gases should also be available on site. The device has been developed for investigation use in research protocols that underwent rigorous review by the local Institutional Review Board (IRB). Under no circumstances should providers operate solely based on the indications included in this manuscript, assembling and using this device without seeking prior appropriate institutional regulatory approval. Starting from the proximal end of the device, assemble the pieces in the following order (**Figure 1**).

1. Building the patient interface

1.1. Take a snug-fitting, standard, non-invasive ventilation face mask of the appropriate size for the subject.

1.2. Connect the mask's built-in elbow port to a high-efficiency particulate air (highly hydrophobic bacterial/viral filter, HEPA class 13) filter through the 22 mm outer diameter (O.D.)/15 mm inner diameter (I.D.) connector.

1.3. (Optional) To facilitate the subject's movement and reduce the risk of disconnection, add a 15 mm O.D. x 22 mm O.D./15 mm I.D. (length 5 cm–6.5 cm) flexible patient connector for an endotracheal or tracheostomy tube between the mask interface and the HEPA filter.

NOTE: Make every effort to avoid leakage of the mask interface. The "patient end" of the device could also consist of a mouthpiece. A nose clip must be added in such a configuration.

2. Building the Y-piece and preparation of the O_2 supply

2.1. Take a 22 mm to 22 mm and 15 F Y-piece connector with 7.6 mm ports. Create the circuit's expiratory and inspiratory limbs on the two distal ends of the Y-piece through two opposite-sense, low-resistance, 22 mm male/female, one-way valves.

2.1.1. Expiratory limb: On one end of the Y-piece, place the one-way valve connector allowing a proximal-to-distal flow only (arrow pointing downward).

2.1.2. Inspiratory limb: On the other end of the Y-piece, connect a one-way valve allowing a distal-to-proximal flow only (arrow pointing upward).

2.2. Connect the proximal end of the Y to the HEPA filter.

2.3. Using standard, kink-resistant, vinyl gas tubing with universal adaptors at both ends, connect the O_2 source to the Y-piece's inspiratory limb. Choose tubing of appropriate length considering the distance between the patient and the source of the gas.

NOTE: The Y-piece connector must have a sampling port on the inspiratory limb. If not, an additional straight connector with a sampling port must be used to supply O_2.

3. Building and attaching the scavenging chamber

3.1. Connect a 22 mm x 22 mm silicon rubber, flexible connector adapter to the proximal end of a scavenger chamber (internal diameter = 60 mm, internal length = 53 mm, volume = 150 mL) containing 100 g of calcium hydroxide ($Ca(OH)_2$).

3.2. Attach a 15 mm O.D. x 22 mm O.D./15 mm I.D., 5 cm–6.5 cm, flexible, corrugated tube to the silicon rubber adapter.

3.3. Connect another 22 mm x 22 mm silicon rubber, flexible connector adapter to the distal end of the scavenger.

3.4. Add the scavenging chamber and tubing assembly to the Y piece's inspiratory limb using a 15 mm–22 mm two-step adapter.

4. Building and attaching the NO reservoir system

4.1. Assemble a 3-L latex-free breathing reservoir bag and a 90° ventilator elbow connector without ports (22 mm ID x 22 mm).

4.2. Connect the other end of the elbow to the central opening of the aerosol T-piece (horizontal ports 22 mm O.D., vertical port 11 mm I.D./22 mm O.D.).

4.3. Attach the T-piece to the scavenging chamber's distal end by advancing it until it fits the silicon rubber connector tightly.

5. Building the NO and medical air supply system

5.1. Build the NO/air gas supply system by attaching two consecutive 15 mm O.D. x 15 mm I.D./22 mm O.D. connectors with 7.6 mm sampling ports and flip-top caps.

NOTE: Once the caps are removed, the sampling accesses will function as gas inlet ports.

5.2. At the distal end of the NO/air supply system, attach another one-way inspiratory valve (arrow pointing upwards).

5.3. At the proximal end of the NO/air supply system, connect a 15/22 mm two-step adapter.

5.4. Connect the proximal two-step adapter to the remaining free inlet of the green T-piece from the NO reservoir system.

6. Attach the air and NO gas flow lines by using standard, kink-resistant, star-lumen vinyl oxygen gas tubing for the following steps.

6.1. Connect medical air to the most distal gas inlet port.

6.2. Connect NO gas from an 800 ppm medical-grade NO tank (size AQ aluminum cylinders containing 2239 L of 800 ppm of NO gas at standard temperature and pressure, balanced with nitrogen; delivered volume 2197 L) to the next port downstream.

NOTE: Tubing must be of appropriate length to reach the gases' sources comfortably. Different tanks or generators of NO can be used as sources of gas.

7. Use in spontaneously breathing subjects

7.1. Set the air, O_2, and NO gas flow according to the desired FiO_2 and NO concentration.

NOTE: The recommended flow rates for administering NO at 80, 160, or 250 ppm are listed in **Table 1** (applicable to 800 ppm cylinders only).

7.2. Position the tight-fitting mask on the patient's face, similar to a non-invasive ventilation interface setup.

7.3. Start the inhalation session for the desired duration.

REPRESENTATIVE RESULTS:

A 33-year-old respiratory therapist working at the ICU at MGH during the surge of ICU admission for COVID-19 volunteered to receive NO as part of the trial involving healthcare workers[15,19]. The trial tested the efficacy of 160 ppm of NO as a virucidal agent, thereby preventing disease occurrence in lungs at risk for viral contamination. The first session of the inhalation prophylaxis was administered before starting a shift through the described device for 15 min. For research purposes, concentrations of inhaled NO, NO_2, and O_2 were continuously measured. NO gas was administered at 3.5 L/min from an 800 ppm gas tank and mixed with air at a flow rate of 15 L/min and an O_2 flow rate of 1 L/min to maintain a FiO_2 at 21%.

The resulting NO concentration was 160 ppm at a total gas flow rate of 19.5 L/min, measured by three standard 15 L/min flowmeters. Oxygen saturation (SpO_2), methemoglobin (MetHb), and heart rate were continuously monitored. SpO_2 remained stable at around 97%. MetHb peaked at 2.3% during NO administration before rapidly returning to the baseline value upon suspension of the gas. The subject did not experience any side effects during or after the session. The NO concentration remained stable throughout the whole period of inhalation. NO_2 peaked at 0.77 ppm and was therefore safely below the recommended toxicity threshold. A representative portion of the recorded tracings of NO and NO_2 signals is depicted in **Figure 2**.

FIGURE AND TABLE LEGENDS:

Figure 1: **Graphic representation of the delivery device.** The single components are indicated in the figure, as named in the text and the **Table of Materials**. The system comprises four major parts: the patient interface; Y-piece and oxygen supply; scavenging chamber; and the NO reservoir system and NO and medical air supply system. Abbreviations: HEPA = high-efficiency particular air; NO = nitric oxide.

Figure 2: **Representative tracing of NO and NO_2 concentrations during the 160 ppm NO inhalation in a healthy healthcare worker.** Abbreviations: NO = nitric oxide; NO_2 = nitrogen dioxide; ppm = parts per million.

Table 1: Setup of NO, O_2, and air gas flows. Gas flows to deliver target NO concentrations at varying FiO_2, as measured with a lung simulator in a bench experiment. NO, and O_2 flow (in L/min) were set to obtain target NO inspiratory concentration (80, 160, and 250 ppm) at the desired FiO_2 (21%, 30%, 40%). A constant medical air flow rate (15 L/min) was used in every setting. A commonly available 800 ppm NO cylinder balanced with nitrogen was used. Abbreviations: L/min: L per min; NO: Nitric Oxide; NO_2 = nitrogen dioxide; FiO_2: Fraction of Inspired Oxygen, O_2: Oxygen; ppm: parts per million.

DISCUSSION:

Given the increasing interest in NO gas therapy for non-intubated patients, including those with COVID-19[8], the present report describes a novel custom device and how to assemble its

components to deliver NO at concentrations as high as 250 ppm. The proposed system is built out of inexpensive consumables and safely delivers a reproducible concentration of NO gas in spontaneously breathing patients. The ease of assembly and use, together with the safety data published elsewhere[16,17], makes this system the ideal embodiment to administer a high NO gas concentration at varying FiO_2 in non-intubated patients. The methodology described herein is currently in use at MGH to investigate the effect of high concentrations of NO to treat, or prevent, COVID-19[14,18,19]. The method can be adjusted based on the local availability of specific consumables, which may differ in brand and size from those described here. Nevertheless, a few critical steps of the protocol must be followed.

The sequence of each gas supply line, the reservoir bag, and the unidirectional valves must not be altered for any reason. A HEPA filter must also be present, particularly in case of any risk of infected bio-aerosol dispersion to the environment. Air leaks might impact the delivery of appropriate NO concentrations. Care should be exercised to use appropriately positioned and sized face masks and to avoid disconnection at any point of the system. The availability of a scavenger chamber with at least the reported amount (100 g) of $Ca(OH)_2$ is also essential to prevent the accumulation of NO_2 and avoid nitric acid formation upon reaction with water in the lungs. The $Ca(OH)_2$ scavenger is designed to undergo a chemical dye reaction upon consumption, functioning as an indicator of its residual absorbent properties. To ensure the efficiency of the scavenger in reducing NO_2 levels, the component should be changed when two-thirds of the canister have changed color. Bench tests showed that NO_2 remained below 1 ppm for the first 60 min and never exceeded 1.3 ppm even after 5 h of exposure to 160 ppm NO[17]. Sessions longer than five hours will likely require the scavenger to be changed.

In case a cylinder is used as a source of NO gas, attention must be paid to the native NO concentration in the tank, as reported by the manufacturer. The NO, air, and O_2 flow settings for a standard NO high-pressure cylinder are reported (**Table 1**). The use of cylinders with different gas concentrations, or alternative NO generating devices[21–23], would impact the flow settings necessary to deliver gas mixtures with the desired NO and O_2 concentrations. NO is diluted in nitrogen as a balance gas in most high-pressure cylinders. The higher the NO concentration, the lower is the net FiO_2 administered to the patient if no supplemental O_2 is added to the mixture. This interplay between NO concentration and FiO_2 must be considered, especially when NO is administered to an already hypoxic patient, or while assessing the efficacy of NO in terms of oxygenation improvement. The resulting SpO_2 increase might be blunted if FiO_2 is not maintained constant during NO administration. Importantly, if no supplemental O_2 is administered, a hypoxic mixture can potentially be generated by mixing high-dose NO and air.

NO has a very favorable safety profile. The molecule's very short half-life further limits the few potential adverse effects. Methemoglobinemia is the most important threat, particularly in the setting of prolonged high-dose exposure because of which MetHb levels should always be monitored closely. MetHb is formed in the blood upon breathing NO by the oxidation of iron present in circulating hemoglobin. Measurements can be obtained through rapid blood testing or non-invasively through SpMet % monitoring. Levels up to 10% are usually well-tolerated in healthy subjects[24]. Hemodynamic deterioration can rarely occur following NO inhalation.

Rebound pulmonary hypertension is another possible risk if the prolonged administration of NO is abruptly interrupted[25]. The device can be modified to sample gas concentrations if needed. A NO/NO_2 sampling access (15 mm straight connector with port) can be placed at the inspiratory limb, before the Y-piece. In that case, to safely add O_2 to the admixture, an additional 15 mm straight connector with a port must be placed upstream and used as an oxygen inlet. However, monitoring the inspired gas concentrations of NO and NO_2 is most likely not clinically feasible because of technical difficulties and the need for dedicated equipment to measure ppm levels of these gases at the bedside. Despite using the same tank, slight variations in the administered concentration might occur, compared to those reported in **Table 1**, based on the patient's minute ventilation. Additionally, standard gas rotameters (0–15 L/min with a stainless steel ball float) do not allow increments smaller than 0.5 L. The availability of high-precision digital flowmeters, similar to those for the setup shown in **Table 1**, would increase the precision of the dose being administered.

The limitations of the described methodology mainly include the scarce data currently available on the proposed device's human use. Although convincingly performing in bench experiments and testing on volunteers and patients[17], to date, data are based on experience limited to a single center[16]. Operators should engage in the use of this novel system and the administration of high-dose NO only if already experienced in the use of NO gas therapy to treat critically ill patients. Depending on the local institutional policy and agreements in force, tanks or other NO gas sources might be challenging to obtain and use as freely adjustable gas sources, outside of the limitations imposed by the delivery devices currently available on the market. NO is an endogenously produced vasodilator[26]. Its administration as a gas therapy is currently approved by the U.S. Food and Drug Administration "for the treatment of term and near-term neonates with hypoxic respiratory failure associated with clinical or echocardiographic evidence of pulmonary hypertension"[27]. However, NO is also routinely used in adults for pulmonary vasoreactivity testing[28] and as rescue therapy in hypoxemic critically ill patients with or without pulmonary hypertension[2,29–31]. The safety and tolerance of a high concentration (160 ppm) of NO have been consistently reported in studies addressing the drug's virucidal, bactericidal, and fungicidal effects[5–7,27]. To administer high-dose NO for research purposes, IRB approval was sought and obtained[14,18,19,32].

To date, the administration of inhaled NO mainly relies on gas tanks and associated bulky machinery. Tank-based delivery devices are commonly designed to administer NO gas concentrations up to 80 ppm. Commercially available systems offer software-based capabilities to deliver an adjustable amount of NO based on the total gas flow being provided to the patient and the desired NO concentration. NO inhalation can be continuous or synchronized with the patient’s inspiration. Measuring NO, NO_2 and O_2 concentrations through an electrochemical sensor cell is always possible. Such expensive devices may offer technical and safety advantages compared to the proposed construction. However, they are expensive and rarely present in more than a few units, being generally used within selected ICUs in intubated patients. As a result, the availability of NO therapy for patients outside the ICU is very limited, even at large institutions. Furthermore, the majority of currently marketed devices do not allow the off-label administration of concentrations higher than 80 ppm. Not surprisingly, by means of currently

available devices, it is virtually impossible to administer NO at high concentrations on a large scale in a limited-resource setting, such as that mandated by a surge of patients and a shortage of medical supplies. Under such circumstances, the need for a simple and inexpensive, yet safe and open-source, device for the administration of this potentially beneficial therapy is critical. This system might be implemented in the future by more investigators and clinicians to safely and reliably administer NO in a reproducible way in COVID-19 and other disease states for which NO properties might be beneficial. In the described methodology, the source of NO is usually a standard gas tank. Other NO sources can be adapted to be used with this delivery system, including tankless devices and generators.

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Figure 1

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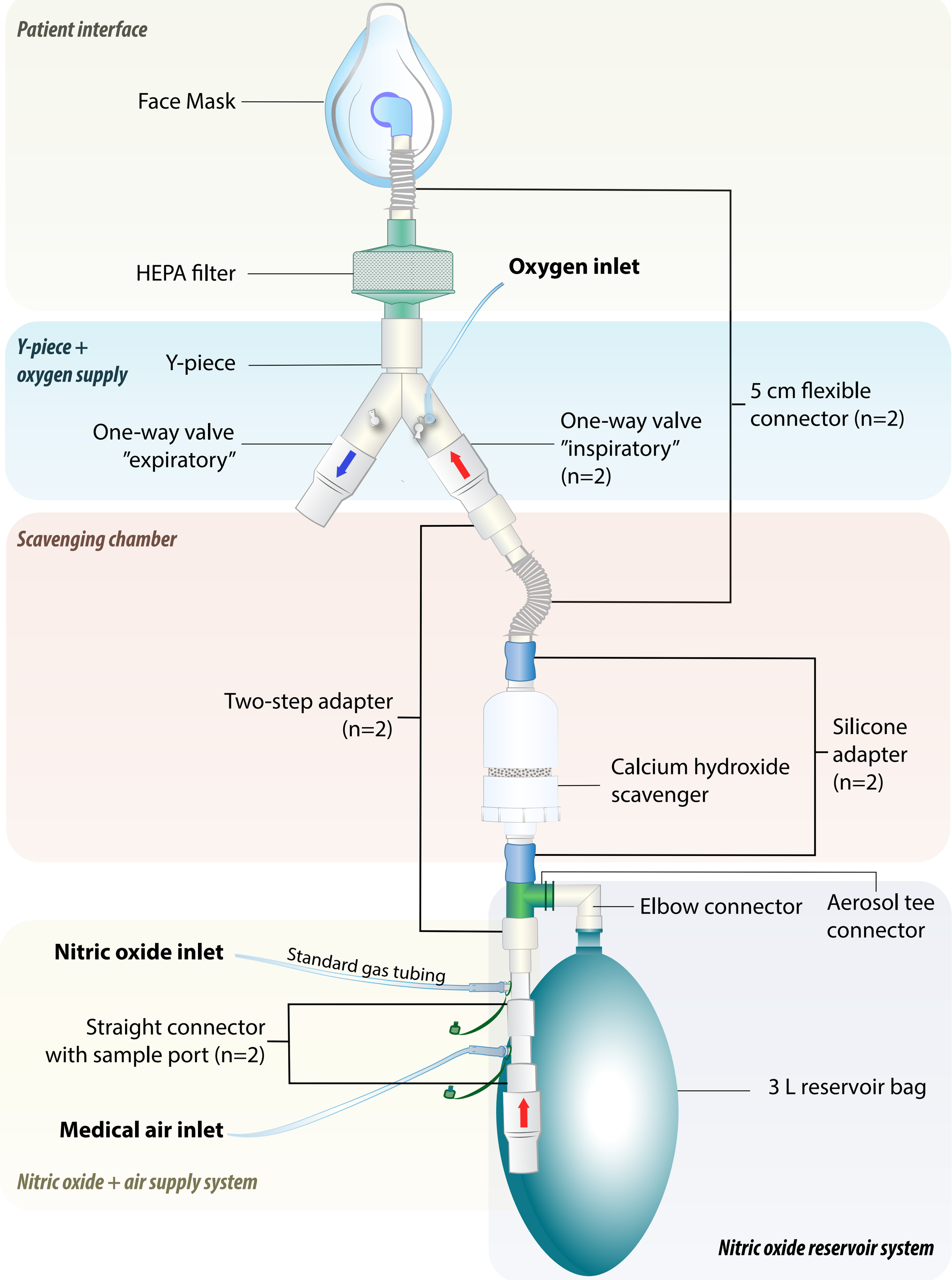

Figure 2

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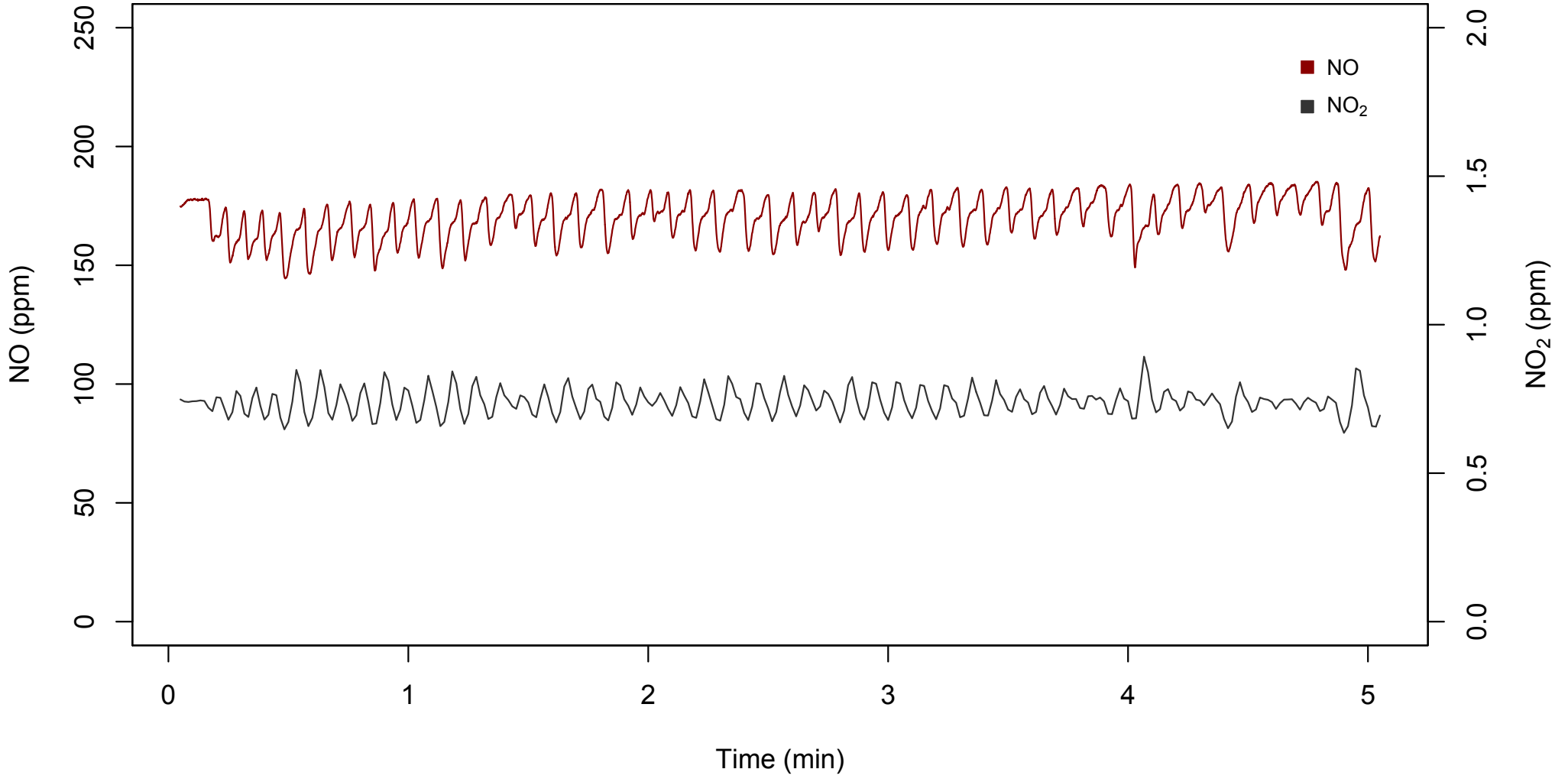

Table 1

Target NO (ppm)	FiO_2 (%)	Flow setup (L/min)			Measured NO_2 (ppm)
		NO	O_2	Air	
80	21	1.67	1.28	15	0.32
	30	1.89	3.28	15	0.32
	40	2.21	7.24	15	0.37
160	21	3.87	1.78	15	0.81
	30	4.38	4.31	15	1.05
	40	5.38	9.59	15	1.2
250	21	6.99	2.1	15	1.57
	30	9.1	7.3	15	2.35
	40	11.91	17.4	15	2.61

Table of Materials

Click here to access/download;Table of Materials;Table of materials.xls

No.	Name of Material/ Equipment	Company
1	90° ventilator elbow connector without ports 22 mm ID x 22 mm OD	Teleflex, Wayne, PA, USA
1	Aerosol tee connector: horizontal ports 22 mm OD, vertical port 11 mm ID/22 mm OD	Teleflex, Wayne, PA, USA
2	Flexible patient connector for endotracheal or tracheostomy tube (15 mm OD x 22 mm OD/15 mm ID, length 5 cm to 6.5 cm)	Vyaire Medical Inc., Mettawa, IL, USA
1	High-efficiency particulate air (highly hydrophobic bacterial/viral filter, HEPA class 13) filter (22 mm ID/15 mm OD x 22 mm OD/15 mm ID connector)	Teleflex, Wayne, PA, USA
1	Latex-free 3-L breathing reservoir bag	CareFusion, Yorba Linda, CA, USA
1	Nitric Oxide tank 800 ppm medical-grade (size AQ aluminum cylinders containing 2239 L at STP of 800 ppm NO gas balanced with nitrogen, volume 2197 L)	Praxair, Bethlehem PA, USA
2	One-way valve 22 mm male/female (arrow pointing towards female end)	Teleflex, Wayne, PA, USA
1	One-way valve 22 mm male/female (arrow pointing towards male end)	Teleflex, Wayne, PA, USA
1	Rad-57 Handheld Pulse Oximeter with Rainbow SET Technology	Masimo Corporation, Irvine, CA, USA
1	Scavenger (ID = 60 mm, internal length = 53 mm, volume = 150 mL) containing 100 g of calcium hydroxide	Spherasorb, Intersurgical Ltd, Berkshire, UK
2	Silicon rubber flexible connectors 22 mm F x 22 mm F	Tri-anim Health Services, Dublin, OH, USA
1	Snug-fit standard face mask of appropriate size	
3	Star Lumen standard medical grade vynil oxygen tubing with universal connectors	Teleflex, Morrisville, NC, USA
1	Straight connector with a 7.6 mm sampling port (15 mm OD x 15 mm ID/22 mm OD)	Mallinckrodt, Bedminster, NJ, USA
2	Two-step adapter (15 mm to 22 mm)	Airlife Auburndale, FL, USA
1	Y-piece connector with 7.6 mm ports (22 mm to 22 mm and 15 F)	Vyaire Medical Inc., Mettawa, IL, USA

Catalog Number	Comments/Description
1641	
1077	
3215	
28012	
5063NL	
MM NO800NI-AQ	
1664	N=2 inspiratory limb (upward arrow)
1665	N=1 expiratory limb (downward arrow)
3736	Including SpMet Option
301-9000	
1115	Variable length according to distance from source of gas. 2.1 m length used in protocol
502041	
1824	
1831	

Response to Review Letter

Dear Editor,

We are pleased to submit the revised version of our manuscript entitled: "**A novel inhalation mask system to deliver high concentrations of nitric oxide gas in spontaneously breathing subjects"** (JoVE61769R1).

We thank the editorial team and the reviewers for the comments and the suggestions, giving us the chance to improve our manuscript. We addressed the points suggested and revised our manuscript.

Below please find our point-by-point responses to both Editorial and Reviewers' comments. Relative modifications to the manuscript text are referenced where appropriate.

Editorial comments:

Changes to be made by the Author (s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling and grammar. Please define all abbreviations at first use, e.g., CPAP (line 22).

Authors' reply: The manuscript has been thoroughly proofread. All abbreviations are defined at first use. The acronym "CPAP" in the summary section has been changed to "positive pressure.".

2. Please provide an email address for each Author.

Authors' reply: A current email address has been added on the title page next to each Author's name.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example (Teleflex, Wayne, PA)-lines 93-94; CareFusion Vernon Hills, IL (lines 102-103); Masimo Rad-57 Handheld Pulse Oximeter with Rainbow … (lines 262-263), etc

Authors' reply: The manuscript has been modified to comply with the journal's requirements. Commercial language has been removed throughout the text.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our," etc.).

Authors' reply: The manuscript has been modified to comply with the journal's requirements. Personal pronouns have been removed throughout the text.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. The Protocol should contain only action items that direct the reader to do something. Please move the Discussion about the Protocol to the Discussion.

Authors' reply: The protocol section has been revised to comply with the editorial standards. Notes have been edited or deleted, with content moved to the Discussion section, when appropriate.

6. Please note that your Protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the Protocol.

Authors' reply: The protocol section has been thoroughly edited to increase precision and provide the highest possible detail level. The proposed setup is a relatively simple sequential assembly. We believe the "how" question can be quickly answered, mainly through the video format.

7. 1 (1.1, 1.1.1, 1.1.2)—please reference Figure 1.

Authors' reply: Figure 1 has been referenced at the beginning of the Protocol section.

8. 1.3: what are the dimensions of these pieces of tubing and connectors?

Authors' reply: The first part of the Protocol (list of items) has been broken down to the appropriate steps in the following sections to precisely report dimensions for every mentioned piece of equipment at the corresponding stages of build.

9. Please move the information in lines 271-275 to the beginning of the Protocol to provide an ethics statement before the numbered protocol steps, indicating that the Protocol follows the guidelines of your institution's human research ethics committee. Also, please provide details about subject selection criteria in the Protocol.

Authors' reply: Details about IRB approval # have been moved to the beginning of the Protocol section as requested. A brief overview of each study's target population in which the device is currently in use has been provided at that same point in the manuscript, as requested.

10. 2.3.4: Please specify dimensions and material of kink-resistant gas tube.

Authors' reply: Tubing material has been specified. Tubing length can be variable according to the distance between the patients and the source of gases. The specific length that will be used in the video (2.1m) has been added to the "Table of Materials", while the specification "Choose tubing of appropriate length considering the distance between the patient and the source of gas." has been added to the Protocol (points 2.3 and 6-Notes).

11. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the Protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

12. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Authors' reply: Following significant editing, the whole Protocol section from point 1 to 7.3 fits into the required 3-pages for essential steps. We believe it all can be considered highlighted and visualized in the video, telling a very cohesive story. Being the Protocol essentially made by subsequent steps of a single build, the logical flow will be very clear. All steps include at least one action written in the imperative tense.

13. Please revise the Discussion to fit into 3-6 paragraphs still covering
a) critical steps within the Protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Authors' reply: The Discussion section has been revised to be structured in six paragraphs covering all aspects required by the editor. Please not that additional words were required to comply with the reviewers' requests.

14. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: Discussion "However, other innovative….home care" (lines 420-423).

Authors' reply: The section has been reviewed and modified. We apologize for the inconvenience.

15. Please number and sort the entries alphabetically in the Table of Materials to make it easier to read.

Authors' reply: The number of pieces needed for each specific part required has been specified, and the items are now sorted in alphabetical order to increase readability.

_____

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The authors present a well-organized description of a self-constructed system allowing delivery of inhaled nitric oxide (iNO) to non-intubated patents. They appropriately identify current logistical challenges with iNO delivery, as well as placing the use of iNO into current clinical context (e.g. possible therapy for COVID-19). The description is detailed and straightforward, if not a little long. The figure accompanying the set up is the most useful portion of the manuscript, and allows a quick understanding of set up being described. A few points, outlined below, if addressed, would add to the manuscript's strength.

Authors' reply: We thank Reviewer #1 for the general comments on our manuscript. To also comply with the editorial requests, the protocol description has been significantly shortened.

Major Concerns:
The major concern for this submission relates to possible regulatory issues associated with the novel delivery mechanism described. The authors should consider a statement regarding current FDA-labeling regarding the delivery of iNO (if it exists). Additionally, the authors should address any regulatory issues that may need consideration if other centers were to utilize the described setup. The authors point out that their use falls under both investigational (IRB-approved) use as well as clinical use. When using a novel set up to deliver an approved medication, does the set up itself fall under any regulatory oversight?

Authors' reply: We appreciate the acute comment on the regulatory body (FDA) that oversees novel devices. To avoid confusion, we edited the manuscript to make as clear as possible that this device has been designed to allow the investigation, under IRB approval, of high-dose nitric oxide, to treat- or prevent- COVID-19. See, among others:

Protocol- paragraph 4: The device has been developed for investigation use in research protocols that underwent rigorous review by the local Institutional Review Board. Under no circumstances should providers operate solely based on the indications included in this manuscript, assembling and using this device without seeking prior appropriate institutional regulatory approval.

Details have also been added on the FDA labeling of NO gas (Discussion-limitations).

Minor Concerns:
The authors might also consider a brief description of troubleshooting the system based on their experience (i.e., where do leaks most commonly occur, where are the "fatigue" points of the system?)

Authors' reply: The Discussion (Par 2- modifications and troubleshooting) has been edited to add further troubleshooting details, including the risk of air leaks and scavenger efficiency.

The authors might also consider a quick statement regarding this current delivery method as it may compare to other investigational delivery systems (such as the iNOpulse) that allow delivery of iNO without mechanical ventilation.

As per JoVE's editorial policy, we could not include any commercial language or comparison with specific marketed devices. However, we hope we can comply with the reviewer's important observation with our revised version of the Discussion section (par 5- significance with respect to different methods) summarizing the advantages and disadvantages of the proposed setup compared to commercially available devices.

Reviewer #2:
Manuscript Summary: In this manuscript, the authors describe a device for inhalation of

high-concentration nitric oxide (NO) gas that does not require mechanical ventilators, CPAP, or high gas flows. This simple and highly adaptable system has been created using standard medical consumables and a snug-fitting mask to deliver NO gas to spontaneously breathing subjects safely. The authors state that it is a safe therapy with few potential risks even if administered at high concentrations. For the authors, inhaled NO gas is routinely used to increase systemic oxygenation in different disease conditions; the administration of high concentration NO also exerts a virucidal effect in-vitro. In addition, no device is currently available that allows easy administration of inhaled NO at concentrations higher than 80 parts per million (ppm), at various inspired oxygen fractions. For authors, the development of a reliable, safe, inexpensive, lightweight, and ventilator-free solution is crucial, particularly for the early treatment of non intubated patients outside of the intensive care unit and in the limited-resource scenario of a pandemic.
Although the manuscript is very interesting in the field of using NO to treat patients with Covid-19. There are some minor comments which should be clarified.

Major Concerns:
None

Minor Concerns:
1. The main side effects of high concentration of inhaled NO therapy should be defined in the text.

Authors' reply: We added the risk for hemodynamic compromise and rebound pulmonary hypertension to the discussion section, next to the already discussed risk of methemoglobinemia. Although not technically a side effect of NO therapy, the risk of NO2 inhalation is a risk we also discussed as a potential safety hazard of high-dose NO administration.

2. How to differentiate the side effects of inhaled NO with high dose with Covid-19-related respiratory symptoms?

Authors' reply: To our knowledge, there are no side effects of inhaled NO whose manifestations would be clinically overlapping with COVID-19 respiratory symptoms. Methemoglobinemia, hemodynamic compromise (rare), and rebound pulmonary hypertension (at rapid weaning) are the three known demonstrated adverse effects.

3. The effect of high dose of inhaled NO on pulmonary endothelial permeability should be awared because high concentration of inhaled NO has a strong effect on vasodilatation.

Authors' reply: Nitric Oxide is endogenously synthesized by vascular endothelial cells and diffuses into adjacent vascular smooth muscle, thereby naturally maintaining the low vascular tone of the pulmonary circulation in standard conditions. When administered by inhalation, it selectively dilates pulmonary vasculature in ventilated areas of the lung. The vasodilating effect of inhaled NO has a rapid onset of action and a short half-life that results in essentially no effect on systemic vessels. The action of inhaled nitric oxide in responders is more pronounced in conditions of pulmonary vasoconstriction, while limited to no effects

at all can be seen in patients with normal pulmonary hemodynamics. This is based on evidence from the last three decades of animal and human research on the drug and our own extensive experience. Relative to high dose administration, in healthy volunteer breathing high dose NO at 160-200 ppm: we did not observe any alteration of hemodynamics or any adverse event.

We also did not see also any alteration of hemodynamics or formation of edema in non-intubated pregnant patients with severe COVID-19 and in a non-intubated hospitalized patient with mild-moderate COVID-19 (217 treatments of 160 ppm of NO for 30 minutes twice per day) (unpublished data).

In a pediatric patient with CF, repeated inhalation of a high dose of NO (140-180ppm) did not alter hemodynamics and did not show any sign or symptoms associated with increased endothelial permeability (Bartley).

Based on our and other observations, we believe that irrespective of dose, NO is a selective pulmonary vasodilator, not affective systemic hemodynamics. In addition, in humans, we did not record any signs or symptoms associated with increased endothelial permeability.

References:

- Ichinose, F., Roberts, J.D., Zapol, W.M. Inhaled nitric oxide: a selective pulmonary vasodilator: current uses and therapeutic potential. Circulation. 109 (25), 3106–3111, doi: 10.1161/01.CIR.0000134595.80170.62 (2004).
- Gianni S, Morais CCA, Larson G, et al. Ideation and assessment of a nitric oxide delivery system for spontaneously breathing subjects. Nitric Oxide 2020; 104–105: 29–35.
- Safaee Fakhr, B. et al. High Concentrations of Nitric Oxide Inhalation Therapy in Pregnant Patients With Severe Coronavirus Disease 2019 (COVID-19). Obstetrics & Gynecology. Publish Ahead of Print, doi: 10.1097/AOG.0000000000004128 (2020).
- Bartley BL, Gardner KJ, Spina S, et al. High-Dose Inhaled Nitric Oxide as Adjunct Therapy in Cystic Fibrosis Targeting Burkholderia multivorans . Case Rep Pediatr 2020; 2020: 1–6.

4. The indications and contraindications of high dose inhaled NO should be defined.

Authors' reply: There are currently no formal indications nor contraindication to the use of high-dose inhaled NO, whose use is limited to research applications to study the molecule virucidal, bactericidal, and fungicidal effects. Safety limits of exposure to increased levels of NO_2 or Methemoglobin have been repeatedly described throughout the manuscript.