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Efficacy and safety of a novel nitric oxide generator for the treatment of neonatal pulmonary hypertension: Experimental and clinical studies

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ABSTRACT

Persistent pulmonary hypertension of the newborn (PPHN) is a complex pathology resulting from a failure of the post-natal reduction in pulmonary vascular resistance leading to hypoxemia. The standard therapy is inhaled Nitric Oxide (NO) improving oxygenation but its availability is limited, especially in hospitals with restricted financial resources. We evaluated the efficacy and safety of a new device generating NO (TAS + *PLUS),* in three experimental piglet models of pulmonary hypertension (PH), and we later tested its application in a pilot study of newborn patients suffering from PPHN.

Piglets with experimentally induced PH showed a decrease in pulmonary arterial pressure (PAP) after breathing NO. Both acute and chronic exposure of piglets and rats did not cause any adverse effect in blood gas levels and biological parameters. A pilot study including 32 patients suffering from PPHN showed an increase in oxygen saturation ($SatO_2$) and partial pressure of oxygen in arterial blood (PaO_2) leading to a decrease of Oxygenation Index (OI) after compassionate treatment with NO from TAS + *PLUS* device.

The device showed effectiveness and safety both in experimental PH and in the clinical setting. Therefore, it represents an excellent alternative for PPHN management in conditions where commercial NO is unavailable.

1. Introduction

Persistent pulmonary hypertension of the newborn (PPHN) resulting from a failure of the post-natal reduction in pulmonary vascular resistance remains an important disease all over the world, and causes considerable morbidity and mortality [1–3]. The main goal of PPHN treatment is the vasodilation of the pulmonary artery to improve arterial circulation and enhance gas exchanges [4,5]. Among the different approaches, inhaled nitric oxide (NO), which is a potent pulmonary vasodilator acting via cyclic guanosine monophosphate (cGMP), efficiently improves oxygenation of a large proportion of infants suffering from hypoxemic respiratory failure [6]. NO decreases the number of invasive therapies such as extracorporeal membrane oxygenation [3]. Therapeutic strategies of PPHN other than NO include potent vasodilators such as Sildenafil acting via cGMP as well as Milrinone and prostacyclins acting via cyclic adenosine monophosphate (cAMP). However, these systemic agents decreasing pulmonary arterial pressure (PAP) are associated with adverse effects, including dangerous changes in systemic arterial pressure (SAP) as compared to inhaled NO therapy whose systemic effects are less pronounced [4,7,8]. However, the high cost of inhaled NO and the complexity of the system for delivering appropriate dosage of NO are important limitations for the use of this therapy in hospital centres with limited financial resources. Recently, few studies have shown the development of investigational devices to generate NO and reported its efficacy in pre-clinical and clinical studies [9,10]. We have developed a study program to evaluate the possibility of using a novel system that enriches atmospheric air in therapeutic amounts of NO in the clinical setting. The three aims of our study were: first to evaluate the efficacy of the device in decreasing pulmonary hypertension (PH) using three piglet models. Second, to test the safety of the gas produced by the device in piglets and rats during short and long-term exposure and evaluation of adverse effects. Third, to test the

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device in a small group of human ventilated neonates suffering from primary or secondary PPHN with indication of inhaled NO therapy by evaluating its effectiveness in rising oxygen saturation ($SatO_2$) and partial oxygen pressure (PaO_2), and reducing the Oxygenation Index (OI).

2. Material and methods

2.1. TAS + PLUS generator of NO and administration

The NO generator INJECT + MATIC TAS + *Plus*® or TAS + *PLUS*® Genève has been developed through a cooperation between INJECT + MATIC (Geneva, Switzerland, www.injectmatic.com) and our research group and manufactured by INJECT + MATIC Genève. In this study, we refer to the device as TAS + *PLUS*. The TAS acronym (Tank-less Anaesthesia System or TAS system) comes from its use for the "anaesthesia" or immobilization of invertebrates for more than 20 years (www.injectmatic.com) [11]. The original device was optimized for clinical use during the past 12 years in order to gain trust in its stability and security [12,13]. The TAS + *PLUS* is an electro-pneumatic device that continuously enriches atmospheric air in NO through an ionising chamber. The amount of NO can be easily regulated to obtain appropriate therapeutic concentrations (Fig. 1A). This device does not require gas tanks since it continuously delivers a pulsed air flow containing regulated NO concentrations ranging from 1 to 100 ppm. NO_2 was found in the range of 1–5 ppm, which was reduced by a column containing calcium/sodium hydroxide at the gas exit. Fig. 1A shows a picture of the TAS + *PLUS* device, whose dimensions are 15 × 10 × 29 cm and weight 2.5 kg. The device is fed by alternating current and consumes 7 Watts. The diagram represents the different parts of the device. NO is mixed with air in the inspiratory branch of the ventilation circuit of the neonate. NO and NO_2 concentrations are monitored using the analyzer of NO − NO_2 "NOXBOX" from Bedfont Scientific Ltd. (Fig. 1B).

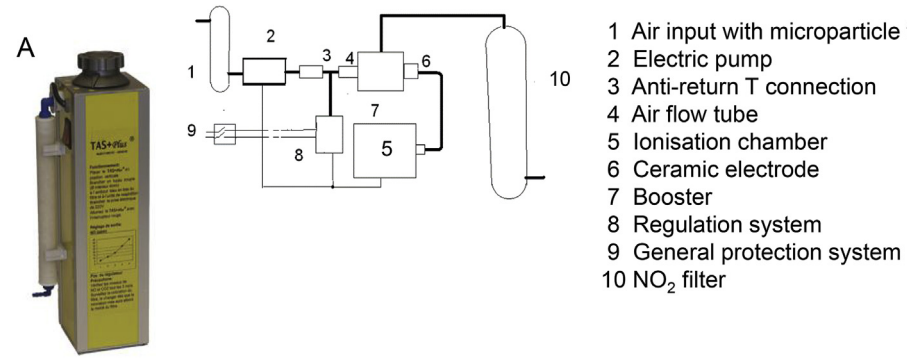
The potential presence of gases other than NO generated by the TAS + *PLUS* device was evaluated by a mass spectrometer Extrel Pentaquadrupole (QMS, Pittsburgh, US) in the ThoMSon Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas (Unicamp), São Paulo, Brazil. This assay confirmed that NO is the only gas generated by TAS + *PLUS* unit and excluded the presence of any other potentially toxic gas (data not shown).

2.2. Piglet and rat studies

These prospective studies were conducted in accordance with ethical animal care regulated by the Honorary Committee of Animal Experimentation (CHEA) from the Republic University and were approved by the Consejo de la Facultad de Medicina, Montevideo, Uruguay with the resolution number 071140-001741-06. The Committee abides by the rules of the American Society of Physiology. The animals received humane care according to standard animal laboratory regulations. These studies included five newborn piglets (*Sus scrofa domestica*) per group of 1–3 days of age (from La Familia farm) and ten Wistar rats of 0 and 24 h of age (from the Faculty bioterium), which is the conventional number of animals used in pig and rat experimental models in the field [14].

2.3. Experimental preparation of piglets

Fifty-five newborn piglets that weighed 1300–1800 g were anesthetized with Ketamine (50 mg/kg of body weight, intramuscularly) and maintained during experiments with intravenous (iv) Propofol (3 mg/kg/h) and Fentanyl (10–20 μg/kg/dose). The animals were intubated following tracheostomy and ventilated with a mandatory assisted mechanical ventilation cycled by pressure as previously described [14]. The ventilator parameters were the following: respiratory frequency 30 cycles/minute, maximum inspiratory pressure 20 cmH_2O, inspired fraction of oxygen (FiO_2) = 0,21, and positive end-expiratory pressure 4 cmH_2O. Central temperature was maintained between 38,5 and 39,5 °C in a servo-controlled open bed. Monitoring included electrocardiogram with DII lead recording, heart rate (HR), $SatO_2$, invasive PAP through pulmonary artery catheterization by thoracotomy, and SAP. These parameters were recorded on an enGuard CM4 monitor (Masimo SET, Ohmeda Medical, IVY Biomedical System Inc., USA)



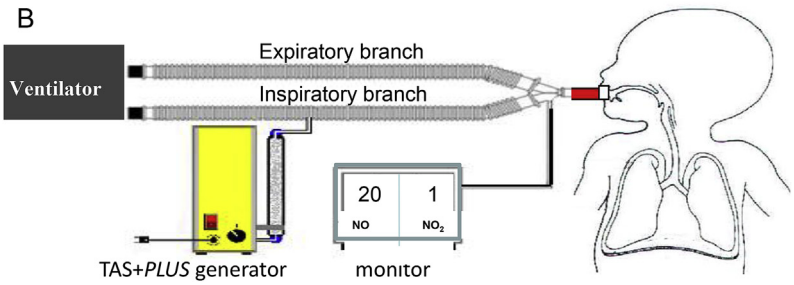


Fig. 1. Schematic representation of the TAS + *PLUS* device generating NO from the air and its connections. A. A picture showing the device and a diagram representing the different parts are shown. B. The device is connected to the inspiratory branch of the mechanical ventilator and the concentration of NO delivered to the neonate is measured with a monitor connected to the Y-piece of the endotracheal tube of the newborn.

connected to a computer (Windaq Software). The results of PAP and SAP are shown as mean pressures (mPAP, mSAP).

2.4. Experimental protocols for piglet pulmonary hypertension (PH)

Forty-five piglets were used in the three models of PH to evaluate the capacity of NO from the device to decrease PAP. The models were the following:

1. Intravenous administration of 200 mg of L-Nitro arginine (LNA), an inhibitor of the nitric oxide synthase (NOS) that induces a failure in endogenous production of NO. LNA-induced PH was established in 20 piglets within 20 min following its administration.
2. Meconium-induced PH: After parameters were stabilized, filtered human meconium was diluted in 20% physiological saline. Meconium was instilled into the airway (2 mL/kg three times reaching a total dose of 6 mL/kg) of 20 piglets as previously described [14]. After meconium instillation, PAP increased progressively during 60 min and was then maintained high for 1 h, during which treatments were tested.

The impact of different treatments other than NO on PAP and SAP was assessed in piglets with LNA and meconium-induced PH. The 20 animals (with LNA and Meconium models) were randomly divided into 4 groups, the first was exposed to NO (10 ppm) from TAS + *PLUS* (n = 5). The second group was treated with Prostacyclin by endotracheal tube (ETT) administration (300 ng) (n = 5). The third group was hyperventilated to obtain blood alkalinisation (pH increased from 7.36 ± 0.08 to 7.46 ± 0.03) by increasing volume ventilation from 0.49 ± 0.09 L/min to 1.06 ± 0.14 L/min (n = 5). The last group was treated with Sildenafil intratracheally (1 mg) [15].

3. The hypoxia model reproducing a moderate perinatal asphyxia was induced in 5 piglets by a mix of air and Nitrogen with a FiO_2 of 0,14 and progressively obtained within 6 min maintaining other ventilation parameters unchanged.

Once the experiments were finished, the animals were killed with 20 mEq of Potassium Chloride iv, and the arrest of myocardial activity was confirmed by electrocardiogram. The duration of every experiment was about 8 h and the laboratory setting worked with up to two piglets a time.

2.5. Evaluation of potential adverse effects upon exposure of TAS + PLUS device in piglets and rats

2.5.1. Piglet model

The general protocol previously described was applied to ten additional newborn piglets divided into two groups: a group breathed 20 ppm of NO generated by TAS + *PLUS* for 12 h and the control group was maintained in ambient air (n = 5/group). Animals were monitored after 6 h and 12 h for the following parameters: HR, blood gas levels, hematology, methemoglobin, renal and liver functions.

2.5.2. Rat model

Forty newborn rats (weighing 5–10 g), 20 controls and 20 exposed to the gas delivered by the device were maintained during 5 days in a plastic chamber specially designed for breathing gas or air while remaining with their mother during the neonatal period. The levels of NO and NO_2 were monitored with NOXBOX® environmental device during the whole period of exposure to NO. Twenty rat pups breathed NO during 5 days. Ten rat pups of each group were sacrificed immediately, and the other 10 rats after 30 days. The analysis included water consumption, growth, neurological motor development (following guidelines already described in Ref. [16]), blood gas levels, hematology, methemoglobin, ions, renal and liver functions. At the end of the experiments, animals were sacrificed by intracardiac injection of Potassium Chloride and macro- and microscopically analysed.

2.6. Nitrate/nitrite determination

Nitrate/nitrite accumulation in the broncho-alveolar lavage fluid (BALF) was measured by the Griess reagent (1% sulfanilamide and 0.1% naphtylethylenediamide in 2,5% sulfuric acid), and optical absorption of samples measured at 550 nm using $NaNO_2$ concentrations as standard as described [17]. These values represented a read out of the exposure to exogenous NO.

2.7. Histologic analyses

Lung, kidney and liver of piglets and rats were fixed with 4% paraformaldehyde, processed, and embedded in paraffin. Tissue sections (5 μm) stained with hematoxylin and eosin (H&E) were evaluated by two pathologists.

2.8. A pilot study in newborn babies of effectiveness in improving respiratory parameters

This prospective study was approved by the ethics committee of the Hospital de Clínicas Dr. Manuel Quintela, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay. The Committee abides by the rules of the Helsinki Declaration. The study was mainly conducted at the Hospital of Clínicas and also in nine different Neonatal Intensive Care Units (ICU) in Montevideo and one in Minas, Uruguay.

2.8.1. Study population

This study was conducted during the periods of July 2009 to March 2016.

Inclusion criteria: written parental consent to receive the proposed therapy; newborn babies older than 34 weeks of gestational age diagnosed with severe refractory hypoxemia receiving conventional intensive care supporting treatment and no access to any other kind of inhaled NO therapy.

Diagnostic criteria of PHT: 1) generalized cyanosis confirmed by $SatO_2$ under 85% which was not improved with oxygen concentration of 100%; 2) pre and post-ductal saturation gradient higher than 5%, without echocardiographic evidence of structural congenital cardiopathy and echocardiographic evidence of PPHN; 3) direct signs of PPHN by measuring PAP and/or indirect signs of PPHN as tricuspid insufficiency, blood flow through permeable ductus arteriosus or oval foramen from right to left, rectification of the interventricular septum, right ventricle hypertrophy, which are defined as a severe PPHN by echocardiography. 4) neonates with respiratory failure severely affected, independently from the gestational age, under conventional or high frequency mechanical assisted ventilation with high parameters (medium airway pressure over 12 cm H_2O), oxygen therapy with high requirements or with an OI [medium airway pressure x FiO_2/partial oxygen pressure (PaO_2)] beyond 20. In all the cases, inhaled NO system was unavailable and due to clinical instability of patients, their transfer was impossible and NO from TAS + *PLUS* was used as compassionate therapy.

Exclusion criteria: Neonates with multiple malformations or malformations incompatible with extra uterine life. Lack of parental consent to receive the proposed therapy.

2.8.2. Data collection

The following parameters were recorded: birth weight, gestational age at birth, age at the beginning of the therapy, treatment or not with Sildenafil, and main diagnosis as established by the health care team. NO and NO_2 concentrations were monitored in real time as previously described (Fig. 1B) [12]. The duration of NO treatment as determined by the physician, depended on clinical improvement of the patient

assessed by haemodynamic and gas parameter improvement.

2.9. Statistical analysis

Quantitative and statistical analyses were performed using Prism 5.0 (GraphPad Software Inc, La Jolla, CA, USA). Variables included in Tables and text are described as means ± standard deviation (SD). In the Figures, variables are shown as median and interquartile range (IQR) with min and max values or Tukey box-and whisker plots. Within-group data were compared using ANOVA followed by Kruskal-Wallis test and with Dunn corrections for repeated measures. Variables with one time point were analysed with Student *t*-test with Welch's correction and Mann-Whitney test. *P* value < 0.05 was considered as statistically significant.

3. Results

3.1. Efficacy of TAS + PLUS supplied gas in the treatment of PH in newborn piglets

This prospective evaluation of the device efficacy used three animal models of PH where piglets before intervention acted as their own control.

The basal conditions of the animals were: HR 140 ± 17 cycles/minute, mSAP 62.2 ± 8.9 mmHg, PaO_2 76.4 ± 9.7, $PaCO_2$ 39.7 ± 1.02 mmHg, and arterial blood pH 7.43 ± 0.03.

L-Nitro arginine (LNA): LNA administration increased mPAP (from basal 25.7 ± 0.7 to Max 42 ± 10 mmHg, $p < 0.05$). In these piglets, inhalation of 10 ppm NO, decreased significantly the mPAP (from 42 ± 10 to 28 ± 9 mmHg Post-NO, $p < 0.05$, 35.7%) after 144 s as shown in Table 1 and Supplementary Fig. 1. LNA-treated piglets also responded to prostacyclin and hyperventilation by decreasing mPAP ($p < 0.05$). In contrast, Sildenafil did not decrease mPAP after 200 s (Table 1). In addition, mSAP increased marginally after LNA administration but was not changed by NO and the other treatments.

Meconium aspiration increased mPAP (from basal 26 ± 5 to 40 ± 6 mmHg). NO exposure reduced significantly mPAP in 111 s (from 40 ± 6 to 31 ± 5, $p < 0.05$). Sildenafil but neither prostacyclin nor hyperventilation decreased mPAP (Table 1).

Following hypoxia piglets showed an increase in mPAP in 6 min (from basal 32 ± 2 to 52 ± mmHg, $p < 0.05$). NO breathing significantly reduced the mPAP (from 52 ± 7 to 33 ± 7 mmHg, $p < 0.05$) after 65 s (Table 1 and Supplementary Fig. 2).

3.2. Safety studies

Safety experiments explored potential adverse effects of high concentrations of NO generated by TAS + *PLUS* in newborn piglets and rats.

Piglet model: Newborn piglets breathed 20 ppm NO or air and analyses were performed after 6 h and 12 h, as represented in Table 2. No significant statistical difference in pH, PaO_2, $PaCO_2$ and HCO_3 between the control and the TAS + *PLUS* group was observed at the baseline, and after 6 and 12 h exposure to NO. In addition, hematological and metabolic parameters, corresponding to liver and renal functions as well as methemoglobin were also similar in the two groups of piglets at baseline and after 12 h breathing NO (Table 2). Nitrates/nitrites from the BALF were increased in piglets exposed to NO from TAS + *PLUS* (control piglets 2.8 ± 0.9 versus 7.2 ± 1.7 μM in TAS + *PLUS* exposed animals, $p < 0.05$) confirming metabolism of NO in the airway fluid.

Rat model: Newborn rats breathed 37 ± 17 ppm of NO and 4 ± 2 ppm NO_2 delivered by TAS + *PLUS* during 5 days and its effects were analysed at day 5 or 30. We did not observe any difference in water consumption and motor development between control and NO exposed rat pups (data not shown). No significant statistical differences were observed in pH, PaO_2, $PaCO_2$, methemoglobin, liver parameters, ionogram, glycemia and blood cell counts between the control group breathing ambient air and the experimental rat group exposed for 5 days to NO delivered by the device. In addition, when the same analyses were done at day 30, no differences were found between the control group and rats exposed to NO (Table 3).

At necropsy of both piglets and rats, macroscopic examination of organs and microscopic examination of lung, kidney and liver on tissue sections did not display any difference between control and exposed animals (Supplementary Fig. 3). Altogether, these data show an absence of toxicity of the exposure to the gas delivered by the device.

3.3. Pilot study: efficacy of NO produced by TAS + PLUS to treat patients with PPHN

During the period of the study, 32 neonates suffering from PPHN were treated with NO from the device. The standard inhaled NO therapy was indicated for our patients, but the Intensive Care Unit did not have any possibility to use it. Admissions were due to primary (10 patients) or to secondary PPHN (22 patients) (Table 4). Secondary PPHN included patients suffering from different morbidities such as diaphragmatic hernia, sepsis/pneumonia, pulmonary hypoplasia, meconium aspiration syndrome, perinatal asphyxia, and pulmonary

Table 1
Efficacy of NO delivered by the device on experimental Pulmonary hypertension (PH) in piglets. Summary of the different treatment outcomes during experimental PH induced by LNA, meconium aspiration and hypoxia. Treatments included NO (10 ppm) from TAS + *PLUS* device, prostaglandin trough ETT (300 ng), hyperventilation, and Sildenafil trough ETT (1 mg). The table shows mPAP at baseline, after induction of PH and post-treatment.

Piglet Model	Treatment	mPAP (mmHg)			mSAP (mmHg)			Decrease of mPAP (%)	Time Post-NO in seconds	P Max m PAP vs Post-mPAP
		Basal	Max	Post-treat.	Basal	Max	Post-treat.			
LNA	TAS + PLUS	25 ± 7	42 ± 10	28 ± 9	56 ± 16	71 ± 14	75 ± 12	35.7	144	$p < 0,05$
LNA	Prostacyclin	20 ± 5	51 ± 12	38 ± 16	70 ± 17	88 ± 12	82 ± 11	26.6	237	$p < 0,05$
LNA	Hyperventilat.	25 ± 6	36 ± 13	22 ± 5	72 ± 25	78 ± 24	56 ± 11	39.2	240	$p < 0,05$
LNA	Sildenafil	17 ± 3	43 ± 12	52 ± 16	45 ± 4	60 ± 9	60 ± 7	0 (+22)	200	ns
Meconium	TAS + PLUS	26 ± 5	40 ± 6	31 ± 5	68 ± 12	58 ± 11	52 ± 11	13.7	111	$p < 0,05$
Meconium	Prostacyclin	22 ± 3	52 ± 8	38 ± 7	64 ± 2	77 ± 37	49 ± 8	27.3	360	ns
Meconium	Hyperventilat.	29 ± 8	45 ± 13	36 ± 6	57 ± 10	48 ± 4	38 ± 5	21.1	149	ns
Meconium	Sildenafil	26 ± 1	37 ± 6	30 ± 3	55 ± 11	57 ± 10	41 ± 5	16.9	240	$p < 0,05$
Hypoxia	TAS + PLUS	32 ± 2	52 ± 7	33 ± 7	68 ± 13	62 ± 11	59 ± 11	36.2	65	$p < 0,05$

Values are represented as mean ± SD (n = 5/group). The decrease of mPAP (% maximum PH vs mPAP post-treatment) and the time required for the beginning of the effect (in second) on mPAP are shown. *P* indicates the statistically significance of the treatments (maximum mPAP vs Post-treatment mPAP).
Abbreviations: LNA L-Nitro arginine, ETT endotracheal tube, mPAP mean pulmonary artery pressure, mSAP mean systemic arterial pressure, PH pulmonary hypertension.

Table 2
Safety of exposure to NO delivered from TAS + *PLUS*. Newborn piglets were exposed to air (CONTROL) or to NO (20 ppm) (TAS + *PLUS*) for 12 h. Parameters were assessed after 6 and 12 h after starting the treatment.

	Baseline	Baseline	6 h	6 h	12 h	12 h
	CONTROL	TAS + PLUS	CONTROL	TAS + PLUS	CONTROL	TAS + PLUS
pH	7,47 ± 0,13	7,40 ± 0,10	7,41 ± 0,05	7,43 ± 0,10	7,44 ± 0,15	7,42 ± 0,14
PaO_2	98,9 ± 26,9	65,3 ± 17,8	85,0 ± 7,0	70,8 ± 5,1	67,1 ± 32,8	71,3 ± 6,9
$PaCO_2$	36,8 ± 14,6	43,1 ± 9,5	38,2 ± 9,2	40,7 ± 15,0	36,9 ± 13,9	42,7 ± 14,8
BE	2,2 ± 3,1	6,1 ± 14,0	6,8 ± 2,5	2,0 ± 3,6	2,6 ± 2,0	2,1 ± 4,6
HCO_3 (mmol/L)	26,8 ± 2,8	20,8 ± 10,8	24,1 ± 2,6	26,1 ± 3,0	24,8 ± 3,4	26,3 ± 4,1
Creatinin (g/L)	0,67 ± 0,37	0,62 ± 0,23			0,51 ± 0,11	0,51 ± 0,23
Lactic acid (mmol/L)	3,4 ± 1,8	2,7 ± 1,6	2,3 ± 1,0	1,8 ± 0,8	2,3 ± 1,2	1,7 ± 0,9
AST (UI/L)	86 ± 2	103 ± 3			28 ± 2	26 ± 6
ALT (UI/L)	27 ± 6	26 ± 4			17 ± 5	16 ± 5
Platelet (cells x10^5/μl)	2,8 ± 0,5	2,5 ± 1,3			2,5 ± 0,7	2,3 ± 0,7
Leucocyte count (cells x10^3/μl)	5,0 ± 3,3	4,9 ± 3,9			6,5 ± 3,2	7,7 ± 3,2
Hematocrit (%)	25 ± 9	24 ± 3	21 ± 7	26 ± 4	22 ± 6	24 ± 2
Methemoglobin (%)	0,62 ± 0,77	0,48 ± 0,74	0,74 ± 0,72	0,76 ± 0,18	0,64 ± 0,4	0,45 ± 0,57

Data are represented as mean ± SD at baseline conditions and after 6 and 12 h of NO. No significant differences were found between piglets exposed to air and those exposed to NO from TAS + *PLUS* in the three different conditions baseline, and after 6 or 12 of NO (n = 5/group).
Abbreviations: PaO2 partial pressure of oxygen in arterial blood, PaCO2 partial pressure of carbon dioxide in arterial blood, BE base excess, AST aspartate transaminase, ALT alanine transaminase.

stenosis, and two babies presented comorbidities (Table 4). Table 5 shows the clinical characteristics of the newborn included in the study. The majority of infants were of gestational age over 37 weeks at birth (n = 25) and only 7 were below the 37th week of gestation. The continuous NO administration started between 1 and 96 h after birth and lasted between 12 h and 15 days. Patients were first treated with 20 ppm of NO and then, doses were adjusted when needed and gradually decreased to 2 ppm before withdrawal. Nineteen babies (60%) survived but 13 (40%) died due to the underlying pathologies. The majority of dead babies presented secondary PPHN including three babies with diaphragmatic hernia, two with comorbidities (one with meconium aspiration syndrome and pneumonia, and the other with

Table 3
Safety of exposure to NO from TAS + *PLUS* during 5 days. Newborn rats were maintained in ambient air (CONTROL) or continuously exposed to 37 ± 17 ppm of NO and 5 ± 3 ppm NO2 (TAS + *PLUS*) during 5 days and analysed at day 5 or day 30.

	5 days	5 days	30 days	30 days
	CONTROL	TAS + PLUS	CONTROL	TAS + PLUS
pH	7,16 ± 0,06	7,16 ± 0,09	7,26 ± 0,08	7,26 ± 0,09
PaO_2	39,4 ± 32,4	39,3 ± 33,2	47,4 ± 16,9	36,6 ± 18,5
$PaCO_2$	69,4 ± 12,4	76,7 ± 14,4	64,9 ± 9,4	69,0 ± 14,9
BE	−4,0 ± 3,6	−2,1 ± 4,1	2,18 ± 3,4	2,83 ± 3,7
HCO_3 (mmol/L)	23,6 ± 3,5	26,7 ± 6,5	28,6 ± 2,9	29,4 ± 3,1
Hemoglogin (g/dl)	8,0 ± 1,3	7,9 ± 0,8	9,1 ± 1,4	9,2 ± 0,8
Methemoglobin (%)	1,0 ± 0,6	0,6 ± 0,6	0,3 ± 0,6	0,5 ± 0,4
Hematocrite (%)	25 ± 4	25 ± 2	29 ± 4	29 ± 3
K (mmol/L)	4,3 ± 0,6	4 ± 0,5	4,4 ± 0,6	4,6 ± 1,0
Na (mmol/L)	141,6 ± 3,4	142,6 ± 0,5	141,4 ± 2,7	140,9 ± 2,5
Ca (mmol/L)	1,2 ± 0,3	1,0 ± 0,1	1,1 ± 0,2	1,2 ± 0,2
Cl (mmol/L)	102,9 ± 3,7	111,3 ± 1,0	102,1 ± 2,6	101,0 ± 2,4
mOsm (mmol/kg)	290,1 ± 6,3	292,3 ± 3,7	290,0 ± 5,2	288,5 ± 4,3
Glucose (mg/dl)	121,5 ± 12,9	129,1 ± 12,6	127,6 ± 20,2	119,9 ± 27,0
Lactic acid(mmol/L)	2,6 ± 0,8	3,1 ± 0,8	2,5 ± 1,3	3,4 ± 1,1
Urea (mg/dl)			50,5 ± 10,3	46,9 ± 9,4
AST (UI/L)			225,2 ± 74,9	358,0 ± 236,0
ALT(UI/L)			106,5 ± 45,3	137,5 ± 114,5
Alcaline phosphatase (UI/L)			1118,6 ± 278,0	1080,9 ± 270,8
γ-glutamyltransferase (UI/L)			8,9 ± 19,4	5,6 ± 4,4
Total proteins (g/dl)			4,7 ± 0,3	5,1 ± 0,5
Albumin (g/dl)			3,1 ± 0,2	3,12 ± 0,2

Data are represented as mean ± SD. No significant differences were found between groups exposed to air or to NO from TAS + *PLUS*, (n = 10/group).
Abbreviations: PaO2 partial pressure of oxygen in arterial blood, PaCO2 partial pressure of carbon dioxide in arterial blood, BE base excess, AST aspartate transaminase, ALT alanine transaminase.

Table 4
Diagnostic categories of the 32 patients included in the study.

Primary PPHN	10
Secondary PPHN	
Diaphragmatic hernia	3
Sepsis/Pneumonia	8[a]
Pulmonary Hypoplasia	1
Meconium aspiration Syndrome	5[a]
Perinatal asphyxia	6[a]
Pulmonary Stenosis	1

[a] Two of the patients presented comorbidities and are included in two categories.

Table 5
Clinical characteristics of the newborn patients included in the study.

Birth weight (g, mean ± SD)	3309 ± 801
Gestational age (weeks, mean ± SD)	37 ± 3
Gestational age < 37 weeks (%)	22
Age at the beginning of the therapy (hours)	24 ± 20
Continuous treatment duration (days ± SD)	4.2 ± 3.2
NO dose in ppm (mean, range)	20 (3,27)
Sildenafil (%)	44
Outcome	
Discharged home (n)	19
Died (n)	13

perinatal asphyxia and sepsis/pneumonia), two with perinatal asphyxia, one with pulmonary hypoplasia, one with pulmonary stenosis, two with sepsis/pneumonia and two out of ten suffered from primary or idiopathic PPHN.

The main respiratory parameters were evaluated before and after breathing NO delivered by TAS + *PLUS*. Evolution of the $SatO_2$ in the group of 32 patients before and after treatment showed a statistically significant increase after 3 h treatment (means ± SD, from 88 ± 9% to 93 ± 8% after 3 h, $p < 001$) (Table 6) which was sustained at 24 h post-treatment. However, three patients with diaphragmatic hernia did not respond to the treatment after 24 h (Fig. 2A). The PaO_2 significantly increased after the first 3 h of treatment (from 55 ± 23 to 99 ± 66 mmHg, $p < 0.05$) and was maintained for 24 h after treatment (Table 6) (Fig. 2B). A decrease of the OI was observed after 3 h of NO exposure and was statistically significant (from 24 ± 12 to 17 ± 10, $p < 0.05$) (Table 6). The evolution of the OI during the 24 h post-treatment showed a significant decrease as compared with the value before intervention (Fig. 2C). Among patients, 14 out of 32 (44%) were treated with Sildenafil, some of them before and others at the end of the NO therapy. Patients who received Sildenafil did not show any differences in demographic characteristics, oxygen requirements, or therapy duration compared with those without Sildenafil. Patients treated with Sidenafil responded to NO in a similar way to those who were not treated and the two groups did not show differences in the evolution of respiratory parameters. We observed a decrease in the OI at 24 h after NO treatment in both groups of patients (Sildenafil group, before NO 19 ± 7 and 24 h after 7.9 ± 4.1 $p < 0.05$; group without Sildenafil before NO 19 ± 5 ant 24 h after 5 ± 2, $p < 0.05$) indicating that the improvement of OI was unrelated to Sildenafil therapy. Blood methemoglobin was evaluated before the NO treatment and values were 0.7% ± 0.3 and at 24 h post-treatment increased to 1.5% ± 0.2. During the first 24 h of treatment, no changes were observed in FiO_2, SAP and pH.

Table 6
Significant changes of the main respiratory parameters (mean ± SD) of newborn babies suffering from PPHN, 3 h before and after NO therapy using TAS + *PLUS*.

Respiratory parameters	3 h before	3 h after
Medium airway pressure (cmH2O)	10,5 ± 3	10,4 ± 3
$SatO_2$ (%)	88 ± 9	93 ± 8**
FiO_2	0.88 ± 0.22	0.94 ± 0.15
PaO_2 (mmHg)	55 ± 23	99 ± 66*
mSAP (mmHg)	49 ± 15	50 ± 18
pH	7,23 ± 0,15	7,23 ± 0,15
OI	24 ± 12	17 ± 10*

Abbreviations: SatO2 oxygen saturation, FiO2Fraction of inspired oxygen, PaO2 partial oxygen pressure, mSAP mean systemic arterial pressure, OI Oxygenation Index.

4. Discussion

The present study aims to assess the efficacy and safety of a low-cost device generating NO that can be applied for PPHN treatment. First we show that experimental PH can be reduced by breathing NO generated by TAS + *PLUS* device demonstrating its efficacy in newborn piglets. Second, acute and chronic NO exposure of animals did not cause any adverse effects as regards blood gas, and biological parameters. Third, a pilot study in patients suffering from PPHN confirmed its efficacy by improving clinical condition.

We report that TAS + *PLUS* device reduced mPAP in the three piglet models and constitutes an effective pulmonary vasodilator therapy. The NO delivered by TAS + *PLUS* therapy showed effects after LNA-induced PH, meconium instillation and hypoxia animal models. However, the meconium model presented higher variability in the response to NO therapy probably due to the persistence of meconium in the airway. The same trend in decreasing mPAP after other treatments was observed, although was not statistically significant except for Sildenafil treatment. Sildenafil treatment was not useful in the LNA model, probably due to the blockade of endogenous NO by LNA. In the three PH animal models, the response to NO was rapid and well controlled. Furthermore, this therapy has the advantage to trigger less adverse effects than those observed with other treatments such as systemic inotropes or hyperventilation [7]. As expected, inhaled NO is a potent and specific therapy for the treatment of PPNH with a rapid efficacy and reduced secondary effects compared to other systemic agents such as Sildenafil [8,18–22].

Previous reports have shown that experimental prolonged exposure to high concentration of inhaled NO in healthy piglets has a transient natriuretic effect [23]. We have considered the possibility of a toxic effect during the neonate period and performed safety studies in neonate piglets and rats. Our data showed absence of adverse effects as regards blood gas and renal and liver biological parameters in both short and long-term exposed animals. We found an increase in nitrate/nitrite concentration in the BAL fluid of exposed piglets, which corresponds to degradation products from NO metabolism and indicates that the animal incorporated and adequately metabolized NO. In conclusion, in conditions of experimental PH in newborn piglets, TAS + *PLUS* exposure showed great efficacy in different types of PH without inducing adverse effects, neither in acute nor in long-term evaluation.

Based on these experimental results, we performed a clinical study in newborn patients in conditions where conventional inhaled NO equipment was not available. As far as we know, this is the first report showing the use of an *in situ* NO generator in newborn patients suffering from severe respiratory failure in compassionate conditions. The majority of the cases included in this study were term newborn and only 22% were preterm as the main indications for NO use are usually pathologies of term babies. The initiation and duration of the NO treatment were in agreement with previous studies using other clinical commercial devices. Assuming that all patients suffered from a severe and complex pathology, and that in our country the availability of commercial NO is very limited, 14 out of 32 patients received another treatment with Sildenafil concomitantly with NO produced by TAS + *PLUS*. Patients who received Sildenafil did not show any difference in responses to NO compared to those without Sildenafil. A majority of patients (60%) survived but (40%) died due to the

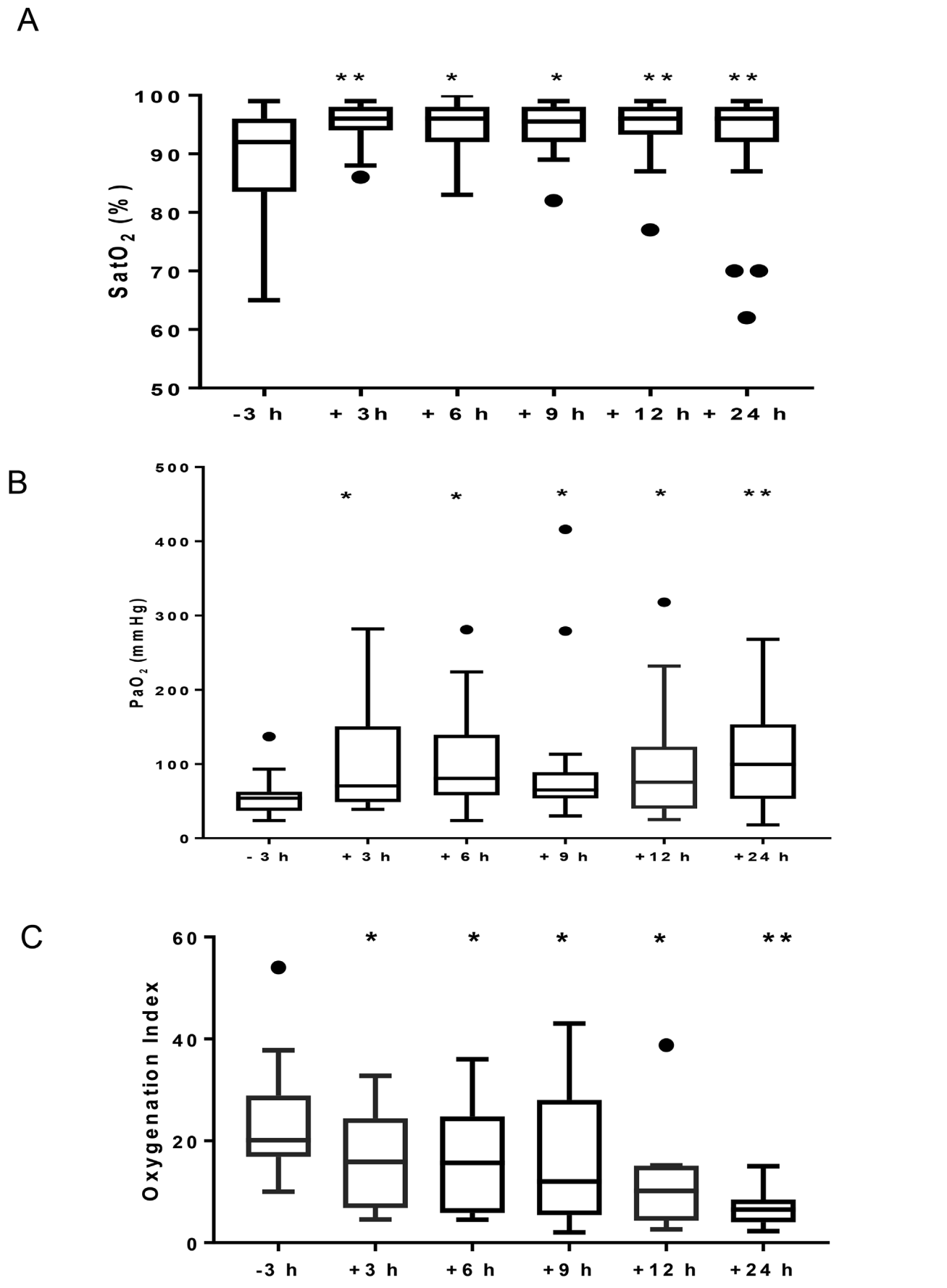


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Fig. 2. A. Evolution of the $SatO_2$ is shown before (- 3 h) and after treatment with NO delivered by TAS + *PLUS*. Values are represented as Tukey box-and whisker plots that show min and max values excluding outliers, box with interquartile range (IQR) (25–75 percentile), median and outliers (black dots) which correspond to more than 1.5 times upper quartile. *p < 0.05 or **p < 0.01 vs 3 h before treatment (n = 32). B. Partial oxygenation pressure (PaO_2) before (−3 h) and the increase after different time points of treatment with NO delivered by TAS + *PLUS* in newborn patients with PHT. Values are represented as in A. (n = 26). C. Oxygenation Index before (-3 h) and its decrease at different time point after breathing NO delivered by TAS + *PLUS*. Values are represented as in A. (n = 16). *p < 0.05 vs 3 h before treatment.

underlying pathologies. The high rate of death among patients could be expected considering the severity of the pathologies of the newborn patients. The pathologies in which the treatment was indicated were related to primary PPHN but also secondary PPHN as a consequence of other morbidities which are well known to induce PPHN including diaphragmatic hernia patients [6]. However, a recent publication has concluded that inhaled NO is effective for term and near-term infants with hypoxic respiratory failure who do not have specific morbidities such as diaphragmatic hernia [24].

Our data showed the immediate responses of patients to TAS + *PLUS* exposure verifying the quick response evidenced by the increase in $SatO_2$ and PaO_2, and a rapid decrease in OI resulting in lower needs of ventilation. This improvement was associated to a systemic hemodynamic stability expressed by no differences in the SAP before and after exposure to NO. Results showed that 3 h after exposure to NO from TAS + *PLUS*, patients exhibited a significant increase in PaO_2 and a reduction of OI. Patient evolution over 24 h of treatment depicted the effect of NO therapy in the large majority of newborn patients. We have previously reported the expansion of the TAS + *PLUS* generator to patients with non-invasive ventilation by applying continuous positive airway pressure through nasal cannula or cephalic carp, and showed good evolution of their respiratory difficulty [12].

Our pilot study in newborn humans as compassionate therapy has some limitations. Our study does not include a control group treated with commercial cylinders of inhaled NO therapy due to the impossibility to resort to this option for purely economic reasons. However, we consider our findings on the effects of NO produced by TAS + *PLUS* as important for the PPHN treatment.

Considering the high price of NO therapy in developed countries, which is a matter of debate [25], and the complexity of the system that restricts the use of this therapy in countries with limited economic resources, innovations to produce new sources of NO may constitute an important way forward. An electric plasma-generated NO device has been tested in six patients with PH and shown pulmonary vasodilatation reducing PAP [10].

TAS + *PLUS* generates NO with very low levels of NO_2, which are acceptable for its use in human ventilation circuits. From a medical point of view, the NO portable generator can be adequate for being used in humans since it can deliver clinical concentrations of NO without toxic contaminants. The use of the TAS + *PLUS* system has many advantages like producing a continuous flow of NO *in situ* and avoiding the need of a complex and costly equipment supply. The small size of the system, its modest energy consumption, and robust design makes it usable in patient transfers or emergency conditions. The low cost of the device, which amounts to the cost of a few days of conventional inhaled NO therapy offers the possibility to be used in locations that cannot afford NO gas supply.

5. Conclusions

In conclusion, our study demonstrates efficacy and safety in piglets and rats as a proof of concept supporting its use in the clinical setting. This novel TAS + *PLUS* device as a source of NO has proven to be efficient for the treatment of PPHN and represents a breakthrough for patients with conventional indications of inhaled NO therapy.

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Conflicts of interest

The authors declare that they have no conflict of interests.

Author's contribution

All authors contributed to data collection and analysis and have reviewed and approved the final manuscript. FB, MM LV, FS, GS contributed to the design of the project, animal and clinical studies and analysis of data. JAAC, CG, PB, IG contributed to data generation and analysis. FB and IG contributed to preparation and approval of the final manuscript.

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Abbreviations

PPHN	Persistent pulmonary hypertension of the newborn
PH	Pulmonary hypertension
NO	Nitric Oxide
TAS	Tank-less Anaesthesia System
$SatO_2$	Oxygen saturation
FiO_2	Fraction of inspired oxygen
PaO_2	Partial pressure of oxygen in arterial blood
$PaCO_2$	Partial pressure of carbon dioxide in arterial blood
mSAP	mean systemic arterial pressure
mPAP	mean pulmonary arterial pressure
OI	Oxygenation Index
HR	Heart Rate
LNA	L-Nitro arginine.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pupt.2018.12.002.

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