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RESPIRATORY DISTRESS SYNDROME: ASPECTS OF INHALED NITRIC OXIDE SURFACTANT AND NASAL CPAP

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-det gælder om att samle sig om ett gott solit utstyr som ikke skall kompletteres-

Peter Wessel Zapffe

Its all about gathering a solid collection of equipment that does not need to be upgraded

To Helena

Abstract

Respiratory distress syndrome (RDS) still represents one of the main problems in the treatment of premature infants. Despite the use of surfactant replacement therapy RDS adds to the need for endotracheal intubation and mechanical ventilation (MV). Enhancing the efficacy of CPAP treatment could reduce the use of MV thereby possibly minimising complications such as bronchopulmonary dysplasia (BPD)

The overall aim was to find methods to reduce the severity of lung damage in newborns by augmenting the efficacy of early treatment by combining nasal CPAP and exogenous surfactant with inhaled nitric oxide (iNO).

We devised an iNO delivery system by modifying a commercially available nCPAP system and measured the NO₂ formation at different stock gas concentrations. We used this system to record the acute effects on oxygenation, respiratory rate and CO₂ levels in 15 RDS infants during a short term exposure to 10 ppm iNO under nCPAP treatment in a cross-over study.

The modified system had a low rate of NO₂ formation. Stock gas cylinder concentration of 50 ppm NO had several advantages allowing simplification of administration and monitoring in comparison with 1000 ppm. Applying the system to premature newborns with moderate RDS, results in significantly improved oxygenation especially in the more premature patients. Respiratory rate and CO₂ levels remain unaffected in spontaneously breathing patients during a 30-min exposure. A secondary aim was to investigate different possible adverse effects of iNO to patients and staff. We measured the occupational exposure levels encountered working close to a baby on iNO-CPAP and described the expected addition of NO and NO₂ to the air in an ICU room. We also investigated the levels produced in case of a sudden release from an NO gas cylinder in an ICU room. The system contributes only to a small extent to ICU room levels of NO_x. As a point source, brief peaks of more concentrated but still low NO levels were seen. The total release of a 20 litre cylinder 1000 ppm NO resulted in room levels of 30 ppm NO and 0.8 ppm NO₂.

Our proposal to use early NO in illnesses characterized by surfactant dysfunction clearly calls for studies of larger groups of patients. It was therefore important to investigate to what extent NO or concurrently produced NO₂ might damage surfactant and at what dose such effects would occur. Sedated spontaneously breathing piglets were given a high NO dose of 100 ppm for 4h or 10 ppm NO₂. The effect of only sedation on surfactant function was studied separately. The exposure to 100 ppm NO in air for 4 hours resulted in slightly impaired surfactant function with higher surface tension of lung lavage fluid compared to control. This was not due to concomitantly formed NO₂ since piglets exposed to only 10 ppm NO₂ had normal surfactant function.

We also exposed sedated piglets under assisted spontaneous breathing to 40 ppm iNO for 24 and 48 h which resulted in no surfactant abnormalities and preserved features in lung tissue histology.

Just as clinical use of exogenous surfactant has moved in the direction of prophylaxis from rescue, after extensive studies, clinical use of iNO may face a similar development with an increased emphasis on lung development and anti-inflammatory action rather than acute improvements in oxygenation and pulmonary vascular tone.

LIST OF PUBLICATIONS

Papers will be referred to in the text by roman numerals

- I. **Lindwall R, Frostell CG, Lonnqvist PA.** Delivery characteristics of a combined nitric oxide nasal continuous positive airway pressure system. *Paediatr Anaesth.* 2002 Jul;12(6):530-6.

- II. **Lindwall R, Blennow M, Svensson M, Jonsson B, Berggren-Boström E, Flanby M, Lönnqvist PA, Frostell CG, Norman M.** A pilot study of inhaled nitric oxide in preterm infants treated with nasal CPAP for RDS. *Intensive care medicine, Accepted for Publication 2005 02 17.*

- III. **Lindwall R, Svensson M, Frostell CG, Eksborg S, Gustafsson LE.** Occupational exposure to NO and NO₂ during combined treatment of infants with nasal CPAP and nitric oxide. *Manuscript.*

- IV. **Lindwall R, Högman M, Linderholm B, Robertson B, Eksborg S, Frostell CG.** High dose nitric oxide alters surfactant function in spontaneously breathing piglets. *Under review.*

- V. **Zhang H, Lindwall R, Zhu L, Frostell CG, Sun B.** Lung physiology and histopathology during cumulated exposure to nitric oxide in combination with assisted ventilation in healthy piglets. *Pulm Pharmacol Ther.* 2003;16(3):163-9.

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LIST OF ABBREVIATIONS AND EXPLANATIONS

aA pO ₂	Alveolar to arterial O ₂ tension difference
Adsorption	The preferential partitioning of substances from the liquid phase onto the surface. In this case surfactant monolayer
BALF	Bronchial alveolar lavage fluid
BPD	Bronchopulmonary dysplasia
CLD	Chronic lung disease, often used for BPD
CPAP	Continuous positive airway pressure
FiO ₂	fraction of inspired oxygen
GA	Gestational age
HMD	Hyaline membrane disease
ICU	Intensive care unit
iNO	Inhaled nitric oxide
iNOS	Inducible nitric oxide synthase
InSurE	Intubate Surfactant Extubate, protocol for exogenous surfactant treatment
MV	Mechanical ventilation
nCPAP	Nasal continuous positive airway pressure
NO	Nitric oxide
NO ₂	Nitrogen dioxide
NOS	Nitric oxide synthase
NO _x	Oxides of nitrogen (collectively)
PaCO ₂	Partial pressure of carbon dioxide in blood in kilopascal, (kPa)
PaO ₂	Partial pressure of oxygen in blood in kilopascal, (kPa)
PEEP	Positive end expiratory pressure
ppb	parts per billion
ppm	parts per million
PSV	Pressure support ventilation
RDS	Respiratory distress syndrome
SP-A	Surfactant protein A
SP-B	Surfactant protein B
SP-C	Surfactant protein C
SP-D	Surfactant protein D
V _v	Alveolar Volume Density
W/D	Wet/Dry lung weight ratio
WBC	White blood cell (count)
WOB	Work of breathing joule/litre (j/l.)
Y _{max}	Maximum surface tension
Y _{min}	Minimum surface tension

1 INTRODUCTION

Respiratory distress syndrome (RDS) still represents one of the main problems in the treatment of premature and low-birth weight infants in the developed world. Despite the use of surfactant replacement therapy RDS significantly increases the need for endotracheal intubation and mechanical ventilation especially in infants below 27 weeks, gestational age (GA) (50). A number of babies do not respond to treatment and develop a more long lasting lung insufficiency often termed chronic lung disease (CLD) or bronchopulmonary dysplasia (BPD). Both of which has several definitions (46; 99; 131), a useful one is continuing oxygen requirement beyond 36 weeks GA.

Nasal CPAP combined with exogenous surfactant treatment has been associated with a reduction in bronchopulmonary dysplasia (BPD) (124). In Scandinavia the current practice is early surfactant installation followed by brief ventilation and extubation (InSurE, Intubate-Surfactant-Extubate) to nasal continuous positive airway pressure (nCPAP) (125; 126). Using this approach a large number of infants needing ventilatory support avoid intubation and a prolonged mechanical ventilation (MV) (50). Enhancing the efficacy of nCPAP treatment could further reduce the need for intubation and MV, thereby minimizing the complications associated with such invasive treatment, e.g. the development of BPD.

Even with exogenous surfactant treatment, many premature infants with BPD have an increased risk of morbidity during childhood (40; 85) and a less favourable motor and cognitive development (20; 108).

Adding inhaled nitric oxide (iNO), which improves oxygenation provided that alveoli have been recruited (90) and selectively reduces pulmonary artery pressure (98), to InSurE treatment could be one method to further improve the concept. There is an increasing amount of animal data and also some human adult studies supporting the notion that iNO in addition can serve as an anti-inflammatory agent in acute lung injury (12; 14; 43; 102; 140; 143). It also acts as a promoter of normal lung development (116; 119). Currently, only one neonatal study focusing on long-term effects associated with anti-inflammatory or lung protective effects of added iNO has been published (106). The study

used the endpoints death and/or BPD in premature infants with RDS after being randomised to either receiving iNO 5 parts per million (ppm) (10 ppm first day) or placebo for a duration of seven days. Both groups had been given antenatal steroids and multiple surfactant doses prior to randomisation and all patients were mechanically ventilated.

Infants treated with iNO showed a reduced incidence of BPD and/or death and also showed a reduction in severe intracranial haemorrhage compared to the control group. The sub-group with an oxygenation index (OI) below 6.94 seemed to benefit the most from iNO-MV. This group of patients would most likely not have been mechanically ventilated in Scandinavia but would rather have been treated with nCPAP. In the light of these findings it would be desirable to design further studies, investigating additional effects of early combination treatment with surfactant, nCPAP and iNO. Certain consequences of such therapy should be investigated namely adverse effects on surfactant function and occupational hazards to staff in the clinics that employ open non-invasive ventilation systems for iNO delivery.

RDS

Cases studies resembling RDS were independently described by obstetricians in England, France and Germany in the second half of the 19th century. In 1929 the Swiss-Swedish researcher Kurt von Neergaard published experiments on air or liquid filled lungs from dogs and pigs. Using pressure-volume loop recordings he showed that on expiration the contribution towards lung collapse of the surface tension in the alveoli was greater than that of the elastic recoil (127). He also measured lower surface tension in alveolar fluids than in serum or saline solution. He expressed his belief that the surface tension of the alveoli is of great importance to the expansion and collapse of the lungs of new born. Unfortunately his work went unnoticed.

In 1954 Macklin published a paper in which he described the presence of an aqueous mucopolysaccharide rich film on the pulmonary alveolar walls which is able to maintain "a constant favourable surface tension (64). Pattle (1955) and Clements (1957) independently described a surface tension lowering film in the alveoli (17; 86). Mead repeated and confirmed Von Neergaard experiments (72) and in a 1959 paper written with Avery showed that surfactant extracts from infants with hyaline membrane disease (excessive elastin deposition in the alveoli) were deficient in surfactant (5).

The first successful continuous positive airway pressure (CPAP) treatment of RDS was published in 1971 by Gregory who reported a drop in mortality to 20 % from an expected mortality rate of 75 % (35).

The usefulness of antenatal steroids to induce lung maturity was pointed out by a study published in 1972 (55). Surfactant replacement was successfully tested experimentally in the early 70's by Enhörning and Robertson (24). This led to and subsequent pilot human studies (32) and later to randomized trials (9; 25; 31).

1.1.1 Incidence and mortality

In a large Finnish study involving 850.000 births the incidence of RDS was 60% at GA 24-25 wk falling to 6% at GA 34-35 wk and displaying an almost linear relationship to GA, at term the incidence had fallen to 0.5 % (67). The Swedish incidence of mortality for the same weeks of prematurity closely reflect these

Finnish data (49). In a recent Danish survey the incidence mortality at discharge was 56 % and BPD at 36 and 40 weeks GA was 16 % and 5 % respectively (51). Mortality and changes in therapeutic interventions for the period between 1991 and 1999 in very low birth weight (VLBW) infants are reported in Vermont Oxford Network Database. For infants with birth weights from 400 (500) -1500 grams the use of antenatal steroids increased from 24 to 72 %, the use of surfactant from 53 to 62 %, the application of CPAP from 34 to 55 % and finally the use of high frequency ventilation increased from 8 to 24 %. This was associated with a concomitant reduction in mortality up to 1995 after which no further improvement has taken place (42).

Chronic Lung Disease (CLD) / BPD as a result of RDS despite golden standard treatment are still common in the more premature infants (99).

1.1.2 Pathophysiology

The view that surfactant deficiency in prematurity is the leading cause for RDS is well founded. However the story has proven to be more complex. The lungs of these infants are both biochemically and structurally immature, without fully formed capillaries and still too few alveoli (118). In premature infants with RDS the turnover of the scarce surfactant is also much slower and interestingly also slowed in full term infants with severe respiratory failure (11). The systems for coping with oxidative stress are still underdeveloped. In regions of the lung with low or uneven distribution of surfactant some alveoli will completely collapse and reopen during each breath (atelectrauma). If MV is initiated, depending on ventilatory modes and setting, strong shear forces (volutrauma) will occur leading to disruption of delicate alveolar structures further enhancing inflammatory reactions (122). This damage also exposes the underlying matrix, which in turn serves as a strong chemotactic stimulus for white blood cells. When activated and removed from the circulation into lung tissue, they release more cytokines. The premature lung is also less suited to transporting water and salts away from the alveoli (6; 88) and more prone to permeability changes. This allows for plasma protein exudation that will in turn lead to surfactant inactivation. A vicious circle is thus established. There is experimental evidence that if this condition is not arrested in time airway

remodelling will occur and the normal alveolar budding initiated by increasing levels of vascular endothelial growth factor (VEGF)— that uses nitric oxide as a downstream mediator (119) for initiating formation of new vessels will be down regulated and alveolar budding will not take place, leading to BPD with fewer larger alveoli. Baboon studies suggest that a lack of or an abnormally low endogenous nitric oxide production plays a central role in BPD. Lung growth and function was almost normalized, and excessive elastin deposition (hyaline membranes), which is highly characteristic of BPD was absent in a group of premature baboons on iNO for 14 days compared to the control group which developed BPD (71). Interestingly in another premature baboon study, brief intubation, surfactant replacement and subsequent extubation to nCPAP (InSurE) treatment for 28 days also normalised lung development (121).

1.1.3 Clinical Definition

According to Hjalmarsson (1981) the following criteria should be met to qualify for the clinical diagnosis of RDS:

- a. Symptoms and signs of acute respiratory illness within 2 hours of birth.
- b. Increasing oxygen demand during the first 24 to 48 h.
- c. Characteristic chest X-ray (reticulogranular pattern and generally decreased air content of the lungs) (41; 50).

1.1.4 Treatment

Modern treatment focuses on prevention by administration of antenatal steroids and early surfactant replacement (117) as well as providing adequate nutritional support. In addition the airways should be stabilised with positive pressure enabling a reduction of tissue inflammation. Improved gas exchange after recruitment of alveoli with surfactant and CPAP can allow for lower fraction of inspired oxygen (FiO_2) thereby reducing oxygen toxicity (91). Indication for intubation and ventilator treatment increases with lower weight, male sex and with need for higher oxygen supplementation (50; 94). To limit the risk for ventilator induced lung injury (VILI) several strategies associated with improved outcome in children and adults and also thought to be beneficial in RDS patients have been adopted (47) (46). Among these modalities the use

of low tidal volumes, permissive hypercapnia, pressure limitation, adequate positive end expiratory pressure PEEP, synchronised breathing, high frequency oscillatory ventilation (HFOV) and real time monitoring of ventilatory parameters deserve to be mentioned.

1.1.5 Longterm outcome

In the first year of life BPD patients have an impaired growth compared to matched control infants and more frequent re-hospitalizations (15). These are mostly due to upper airway infections such as RS virus infections combined with obstructive symptoms (85). Cognitive abilities both at 3 years of age (108) and at school age are impaired to such an extent that special educational support may be required in 30-49% of BPD survivors (20; 66; 109).

1.2 SURFACTANT

1.2.1 Chemistry

Surfactant is produced by the alveolar type II cells in the lung and forms a very thin film at the interface between air and fluid in the respiratory tract. Surfactant from the alveolar lumen consists of approximately 85-90% lipids, 10% protein and 2% carbohydrates. 80-90% of the lipid weight is phospholipids. 75% is phosphatidylcholine (PC), the anionic phosphatidylglycerol (PG) accounts for ca. 8%, 5% is phosphatidylethanolamine, Almost half of the PC content is dipalmitoylphosphatidyl-choline (DPPC), the principle surface tension reducing compound (19). PC is found to be especially low in RDS (39)

Four surfactant specific proteins have been identified, named in order of their discovery: SP-A, SP-B, SP-C and SP-D. SP-A and SP-D are members of a family of immune proteins known as collectins, or collagen-like lectins. SP-A and SP-D are water soluble while SP-B and SP-C are strictly hydrophobic (48).

SP-B is a hydrophobic tightly folded dimer with three intrachain disulfide bridges. Due to the α -helical secondary structure these bonds become internalized and protected, forming a very stable molecule.

SP-B greatly enhances the stability of the phospholipid membrane and thus the spread of phospholipids into the monolayer. During compression of the alveoli

at a higher surface pressure, SP-B is squeezed out of the monolayer, during expansion, a new cycle is started by SP-B- aided insertion of phospholipids into the monolayer speeding up absorption

SP-C is a highly hydrophobic protein linked with two palmitic acid groups with disulphide bridges at two cysteine residues making SP-C a proteolipid and adding to its lipophilic properties. The protein seems to orient itself almost parallel to a phospholipid monofilm but reorients itself transmembranely in multilayer (132). The loss of one or several of its palmitic groups may change its properties (28). As with SP-B it can be squeezed out of the film during cyclic compression. SP-C is able to stimulate insertion of phospholipids out of the sub phase into the air-liquid interface (83) Apart from the important surface active properties recent studies also suggest an immunoactive role (4).

Genetically inherited deficiencies in the production of surfactant proteins has been identified (82). Lack of SP-B production is lethal in humans with progressive lung injury over months and directly lethal in knock-out animals. Partial production of SP-C in mice decreases resistance to oxygen toxicity. Lack of or inappropriately produced SP-C leads to a slow onset of interstitial pneumonitis that can become symptomatic from 3 month of age to late in life.

SP-A is a large, water soluble and complex glycoprotein consisting of subunits that associates with lipids. SP-A is also considered to play a biophysical role (a) interacts with lipids and affects their reorganization, as a regulator of phospholipid insertion into the monolayer. (b) binds a high affinity receptor on alveolar type II cells, (c) regulates lipid secretion by these cells, and (d) regulates lipid uptake and recycling by these cells.

SP-D is a hydrophilic collagenous glycoprotein a clear surfactant function-related role for SP-D has not yet been identified. Only a small part of SP-D (less than 10%) is associated with surfactant and SP-D is not exclusively found in the lung.

Increasing evidence indicates that SP-A and SP-D as other collectins are important components of the innate immune system of the lung. They interact with various bacterial species and pathogen-derived components. They act as opsonins by binding and agglutinating some pathogens and by presenting them to macrophages elicit phagocytosis. SP-A and SP-D can however both enhance and inhibit the production of reactive oxygen species ROS (100) and nitric oxide (138).

1.2.2 Outcome of exogenous surfactant treatment

In several meta-analyses (112; 113; 117) surfactant versus standard care lowers mortality, incidence of pneumothorax and pulmonary interstitial emphysema with negligible effect on the incidence of BPD (112). Prophylactic versus selective use of surfactant further reduced the relative risk of mortality, pneumothorax and pulmonary interstitial emphysema. No effect on BPD was seen (113). Early surfactant and extubation to nCPAP versus selective surfactant reduced death prior to 28 days of age. Furthermore it minimized the use of MV, reduced necrotizing enterocolitis (NEC), air leak syndromes and intraventricular haemorrhage / periventricular leukomalacia. However no effect on BPD was seen (117).

It is of interest to note a renewed interest in surfactant treatment of acute lung injury and acute respiratory distress syndrome (ARDS). Recently a randomised trial employing multiple doses of calfactant for treatment of infants, children, and adolescents with ALI showed improved oxygenation and lowered mortality compared to controls(136). However length of stay in the ICU or days on ventilator were unaltered. Several previous trials have failed to demonstrate improved outcome with surfactant (36; 114). A contributing factor to these failures could be that treatment was provided too late, in fact at a stage of disease when the lung was simply irreversibly damaged with widespread and severe inflammation. There is also a greater degree of inactivation of surfactant in the older lung with ARDS compared to RDS.

1.3 nCPAP

Gregory introduced the first useful CPAP treatment of RDS with positive pressure applied to an endotracheal tube in 18 infants. Two were given CPAP with the help of a chamber around their heads. A pressure up to 16 cmH₂O was used, resulting in a 37.5% reduction in oxygen concentration in twelve hours. The mortality rate was 20% at discharge against the expected 80% at the time (35).

These results were so compelling that the use of CPAP was never subjected to a randomized clinical trial and when the initial success was not repeated at all institutions in the years to follow less frequent use of CPAP treatment in particularly in the United States was the result. CPAP maintained a foothold in the Scandinavian countries possibly because of the use of better delivery systems (8; 44; 50; 54; 63; 80).

The adequacy of the CPAP technique primarily depends on the effect on lung recruitment and also on the reduction in work of breathing (WOB), verified in the degree of variability of the mean airway pressure. Furthermore, the ability to compensate for leaks, if they occur in the nasal prongs or through the mouth is essential for effective CPAP treatment. The patency of the seal around the nares in early systems was poor. Variable flow devices, such as the Infant Flow and the Benveniste valve used in Scandinavia, have been shown to better accommodate the prerequisites above than constant flow systems (18).

1.3.1 Physiology

Administration of CPAP to premature neonates increases their functional residual capacity (FRC) and stabilises larger airways, thus keeping a larger population of alveoli open throughout the breathing cycle. Seen from a statistical point of view, more alveoli participate in a breath. Apart from promoting an increased surface area for gas exchange this in turn reduces shear forces, limiting progressive damage and inflammation (123).

1.3.2 Therapy with CPAP

In Scandinavia the majority of RDS cases needing ventilatory support are managed entirely on nCPAP throughout their hospitalisation. Only infants with a GA < 27 wk MV are considerably more prone to require ventilatory support (50). Several systems for the administration of nasal CPAP are presently commercially available. Also home built solutions such as 'bubble-CPAP' are used clinically. The important difference between these systems is in performance, especially regarding the ability to cope with leakage at the nose and stability of positive pressure. These parameters greatly influence WOB. Such differences may account for divergent treatment success rates and

subsequent need for MV. The Infant Flow™ (74) presently has the lowest WOB and the widest envelope of use (44; 54). Presently, no human randomised study clearly shows a reduction of BPD from the combined use of CPAP and surfactant (23) resembling the findings in animals (121).

1.4 NITRIC OXIDE

The endothelial-derived relaxing factor (EDRF), seen as an endogenous short range messenger-molecule, was identified as nitric oxide (NO) in the late 1970's and early 80's (3; 33; 45). NO was first described by the groups of Ignarro and Furchgott, with actions of the second messenger cyclic guanosine-3',5'-monophosphate (GMP) investigated by Murad et al (3). The discovery of L-NMMA (L-NG-monomethyl arginine) as the first useful competitive blocker of endogenously formed NO, by the Moncada group (84) added to the rapid research advances in this field.

NO activates soluble guanylate cyclase to form more cGMP, which in turn promotes a calcium dependent relaxation of the smooth muscle cell. The majority of nitric oxide produced is rapidly bound to and inactivated by hæme-groups, giving it a short half-life of a few seconds and an action range of millimetres in tissue. Endogenous NO is produced by a group of enzymes, nitric oxide synthases (NOS), by converting L-arginine to L-citrulline. Several isoforms exist and the enzymes can be constitutively expressed as in the vessel wall – endothelial constitutive nitric oxide synthase (eNOS), being always active having a low nanomolar NO output and involved in regulation of vascular tone; in contrast to inducible NOS (iNOS) which become active after challenge and displays a high temporary NO micromolar output. INOS is present in many types of cells. Activation of iNOS has been associated with super oxide production and inflammation, as well as cellular damage.

NO is found to have a multitude of effects in various tissues and has a variety of biological functions including smooth muscle- relaxation, neurotransmission, immune regulation, cellular differentiation, host defence (130) and regulation of cell oxygen consumption through effects on mitochondrial respiration (10; 75). In the respiratory system NO can be measured in the low parts per billion (ppb) range (37). Endogenous NO production in the respiratory system is increased in response to inflammation (61), as in poorly controlled asthma (133). NO as

an inflammatory marker is not unique for the lungs. Marked increases in NO production can be measured from the intestines in inflammatory diseases as well as infectious gastroenteritis (27).

NO can also be shown to inhibit platelet adhesion and aggregation *in vitro* (92; 93). Through several mechanisms, most importantly downregulation of NF κ B production NO inhibits leukocyte adhesion (130). NO modulates apoptosis and proliferation, being a downstream mediator to VEGF (119).

1.4.1 Therapy with inhaled Nitric Oxide; iNO

In 1991 it was reported that iNO in concentrations of 5-80 ppm, could act as a selective pulmonary vasodilator in lambs when the lung vascular bed was precontracted (30). An intense research effort and early clinical use of iNO in the intensive care setting followed, especially since Rossaint et al described that iNO could improve oxygenation in patients with ARDS (98) However it has been difficult to demonstrate significant improvement in outcome, and subsequent randomised studies in ARDS were negative (62; 120).

Since iNOS has been shown to be activated in sepsis and considered to contribute to the vascular hyporesponsiveness to inotropic stimulation experimental treatment of sepsis, based on blocking excessive NO production, has been attempted. However, the result of a large recent study proved paradoxical with increased mortality in the NOS blocked group (59).

In neonatology iNO had impressive effects on oxygenation in infants with severe hypoxaemia due to persistent pulmonary hypertension of the newborn (PPHN) (21; 52; 95). The clinical impression of better outcome could be substantiated by prospective, randomised multi-centre studies giving evidence of a reduced need of extra corporeal membrane oxygenation (ECMO) in the arm using iNO. iNO was registered as a drug in the US (1999) and later in Europe (2001), on the indication severe hypoxaemic respiratory failure with the aim to reduce the need for ECMO (16; 65; 81; 111).

There is also emerging evidence that administration of NO leads to reduced levels of several proinflammatory markers such as NF κ B (12) thought in turn to influence severity of inflammation (139). Nitric oxide is utilised in white cell internal signalling for adhesion, rolling and chemotaxis. At high concentrations (>2 μ mol / l at cell level) nitric oxide will stop white blood cells(WBC) from

retracting roll receptors and reduce the numbers of WBCs entering the lung (29; 53; 102). This adds to a rationale for investigating earlier application of iNO in RDS.

1.4.2 Toxicity of nitric oxide

The toxicological effects of iNO (79; 134) can be classified into NO derived effects e.g. the direct toxic effect of NO in biological systems and those of concomitantly formed higher oxides of nitrogen especially nitrogen dioxide (NO₂). iNO has to diffuse through the surfactant layer and by doing so may cause direct damage to surfactant components or to its metabolism. Nasally produced NO in newborns can reach concentration of up to 4.6 ppm during occlusion of the nares (104). Concentrations exceeding 20 ppm NO have been measured in gas aspirated from the paranasal sinuses (60) in adult volunteers. After inhalation and absorption, iNO is inactivated mainly by binding to haemoglobin, forming methaemoglobin (inactive haemoglobin not capable of transporting O₂). Inadvertently high concentrations of iNO can exceed the capacity of methaemoglobin reductase, leading to clinically significant (34) methaemoglobinaemia (135). In newborns this is a particular concern as levels of this enzyme can be low, especially in premature infants (96; 97). Emerging evidence suggest that some NO molecules are not inactivated in this classical way, but instead bind to carrier-molecules such as lipids (56) and nitrosylated proteins (115). In this context it is interesting to note that healthy volunteers show modified kidney function during exposure to iNO with a temporarily impaired sodium excretion (137). However the molecular mechanism for this observation is presently not known. The in vitro anticoagulative action of NO has not been possible to reproduce in short term randomized blinded studies in which iNO was given to volunteers (2).

A large literature exists on the detrimental effects on lung function caused by oxides of nitrogen as air pollutants. Some work has been directed at effects of NO, others have investigated higher oxides of nitrogen (78; 128; 129). Matalon et al exposed tracheotomised newborn lambs to increasing doses of iNO in either 21% or 60% oxygen (69). Their results clearly demonstrated a capacity for iNO to impair surfactant function at a dose of 80 ppm or more.

Concomitantly formed NO_2 was reported as remaining below 2 ppm, but a control experiment with only NO_2 exposure was not performed.

The formation of NO_2 during iNO is dependent on both oxygen and NO concentration. The rate of oxidation of NO to NO_2 is proportional to the cube of the pressure, the square of the concentration of NO, and linearly proportional to the concentration of oxygen (103). Thus uneven distribution and mixing of different concentrated gases, might result in higher levels of NO_2 formation at a given final concentration of NO, if the source of NO is more concentrated (103). For example if 20 ppm final concentration is achieved using a stock-gas cylinder of 100 ppm NO in N_2 , it is possible that formation of NO_2 is lower, as compared to when the same final concentration of 20 ppm NO is achieved by using 1000 ppm NO in N_2 stock cylinder gas (103).

2 AIMS

The overall aim was to find methods to help reducing the severity of lung damage in newborn infants and to reduce the development of BPD by augmenting the efficacy of early treatment by combining several already existing therapies (nCPAP and exogenous surfactant with iNO). A secondary aim was to find and describe possible adverse effects on the patients and staff, during exposure to iNO using non-invasive open systems.

More specifically we wanted:

-to create and validate a combined system for iNO + nCPAP delivery, and examine levels of NO₂ formed in such a delivery system (I).

-to study the short-term effects on gas exchange of the combination of iNO and nCPAP in a pilot study on moderately premature infants with RDS (II).

-to study the contribution of an iNO-nCPAP system to background hospital room levels of oxides of nitrogen (NO_x) as well as studying the same system as an occupational point source for the staff attending a child during iNO treatment in an incubator. (III).

-to create an animal model allowing for separate inhalation of a high concentration of either iNO or NO₂ during spontaneous breathing, in order to examine the effects on surfactant function (IV).

-to study effects of prolonged iNO exposure (24-48h) on surfactant function and lung morphology, in a model with healthy animal aiming to minimize confounding factors by using minimal ventilatory support and low inspired oxygen fraction. (V).

3 MATERIALS AND METHODS

3.1 SUBJECTS (II)

Ethics + Medical products agency permits were sought and given for the study (KI regional ethics committee NO: 01-211) and (MPA Dnr:151:2001/62934) We included 15 moderately ill neonates treated with nasal CPAP to receive study gases. The population had a median gestational age of 32 (range 27 - 36) weeks, median birth weight was 1940 (1100 - 4125) grams and median postnatal age at study start was 23 (3-91) h.

3.2 ANIMAL MANAGEMENT (IV, V)

Ethics permits were sought and given for the studies; **(IV)** from Tierps Tingsrätt (permits c-239/95, c-342/95 c-190/97) and **(V)** from ethic committee of pediatrics, Fudan university Shanghai (permits 2000-01-01).

Thirty-three pigs of Swedish landrace **(IV)** and sixteen Chinese male piglets **(V)**, both 2-4 weeks old were studied. They were given premedication and disassociative anaesthesia while preserving spontaneous breathing. Azaperone and glycopyrron bromide was administered as premedication in study **(IV)**. Anaesthesia was induced with a mixture of tiletamine/zolazepam/medetomidin given intra muscularly. Maintenance of anaesthesia was achieved with ketamine and azaperone. At the end of the protocol, animals were sacrificed by i.v. barbiturate overdose and the animal lungs were lavaged.

In study **(V)** the pigs were premedicated with diazepam and anaesthesia was induced with a mixture of ketamine and sodium hydrobutyrate. Maintenance was with ketamine and diazepam. At the end of the protocol, animals were sacrificed by 10–20 ml of 10% potassium chloride i.v. after heparinization and the animal lungs were processed.

3.3 PAPER I

We used a modification of the commercially available Infant Flow CPAP system (Fig 1, 2a-d; Electro Medical Equipment, Brighton, UK). It was chosen because of stable pressure and high flow resulting in low work of breathing (WOB) for the child (54; 74). The constant fresh gas flow of this system is a desirable characteristic when adding nitric oxide, as the mixture is kept constant. Two concentrations of NO stock gas were used; 50 ppm and 1000 ppm in nitrogen (AGA AB, Lidingö, Sweden). The two channel mass-flow controller Nomius (Dansjö Medical AB, Stockholm, Sweden) (fig 5) was used to deliver NO stock gas into the mixing chamber as well as air-oxygen. A gas mixer (Siemens-Elema AB, Solna, Sweden) was used to blend air and oxygen upstream of the second channel of the mass flow controller

NOxBOX, a fuel cell device (Bedfont Scientific, Rochester, Kent, UK), previously validated for monitoring of NO and NO₂ (103), measured NO/NO₂. The sensors were calibrated at regular intervals with gas containing 84.5 ppm NO and 7.1 ppm NO₂ (AGA AB). Measurement was linear over the target range. A pump was used to avoid pressure fluctuations and reduce formation of NO₂ by shortening transport time through the sampling system.

The paramagnetic oxygen sensor of a CS-3 monitor (Datex-Ohmeda, Helsinki, Finland) measured the FiO₂. It has several advantages compared to oxygen fuel cells: a fast response time and excellent linearity (73) unaffected by NO (personal communication from the inventor: P. Merilainen, Datex-Ohmeda). Pressure in the sampling line was reduced from 70 to 15 cm H₂O by means of a thin Teflon tube to bring this instrument within measuring range.

A custom made mixing chamber (Fig 3, 4) was inserted in the inspiratory limb of the circuit, 27 cm proximal to the nasal prongs. It was designed to create turbulence, to ensure adequate mixing of NO with the fresh gas flow thus avoiding laminar flow prior to measuring (110). A mid-stream sampling port was located at the downstream end.

Two sets of experiments were carried out. Firstly, 1000 ppm NO in N₂ stock cylinder gas was diluted with oxygen/air to a final concentration of 2-100 ppm NO at a FiO₂ of 0.8 and a CPAP of 5 cmH₂O. Secondly, 50 ppm NO in N₂ stock

Fig 1, The system has no moving parts. Pressure measurement channel (A). Compressed air jets (B, darker blue) enters the breathing channel (C, light blue) slightly offset. Due to this a low-pressure area forms giving rise to adherence between the wall and the jet (the Coanda effect). The low pressure will change its position during inspiration and expiration giving the system both a stable inspiratory positive pressure at a higher inspiratory

flow than a jet entrainment device as well as a stable PEEP during expiration. This is known as a “fluidic flip”

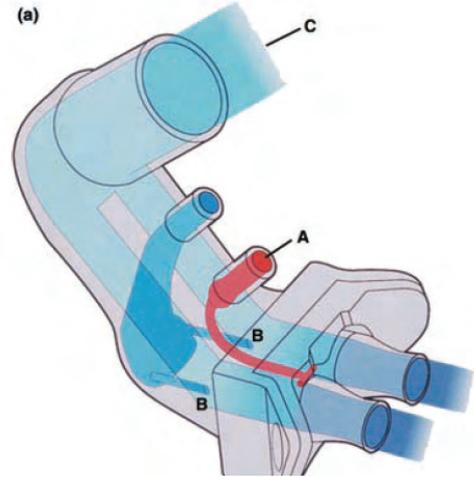


Fig 2a, Inspiratory flow

Fig 2b, Inspiratory pressure

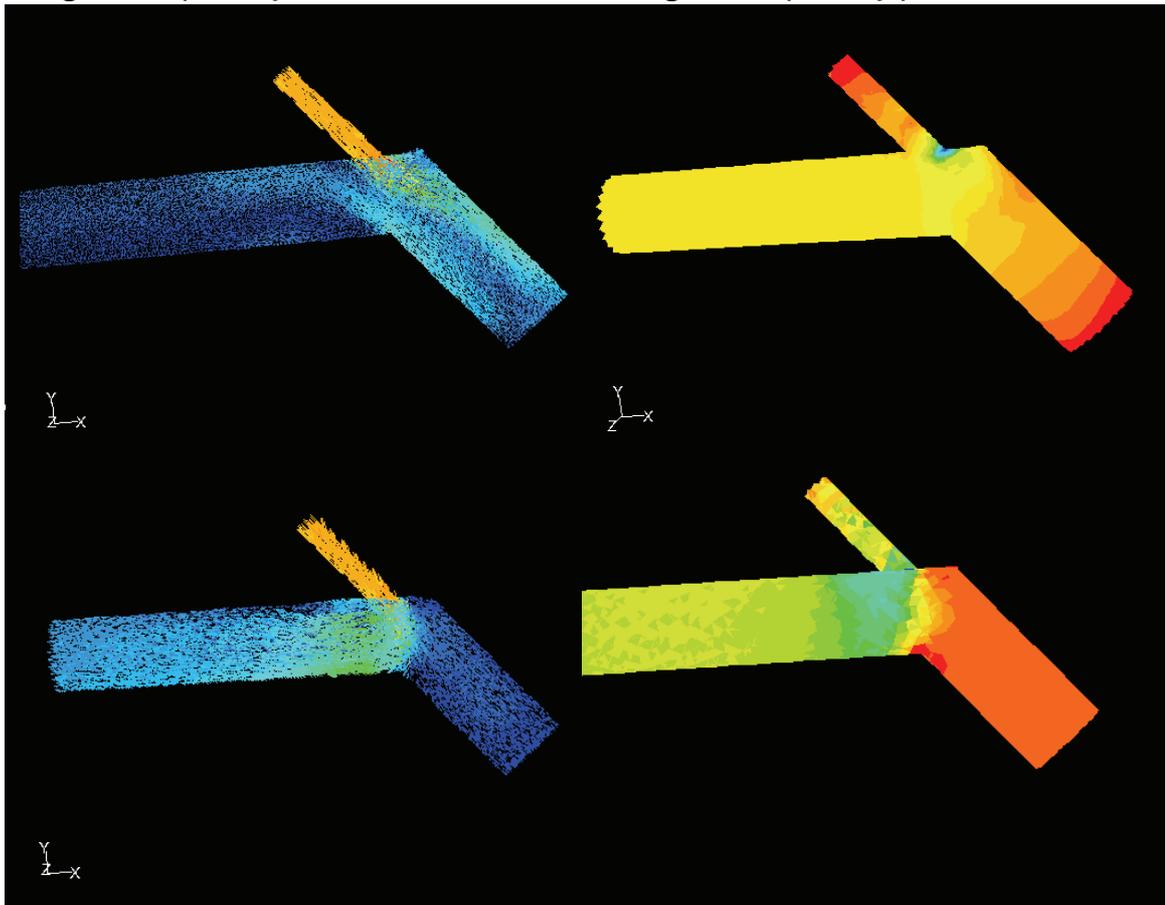


Fig 2c, Expiratory flow

Fig 2d, Expiratory pressure

Fig 3, Mixing chamber

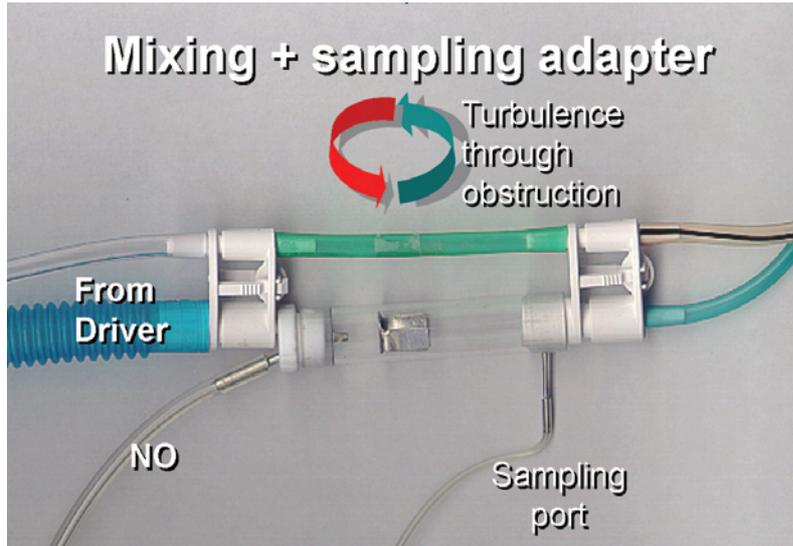


Fig 4, demonstration of mixing by turbulence (b) versus incomplete mixing with laminar flow (a)

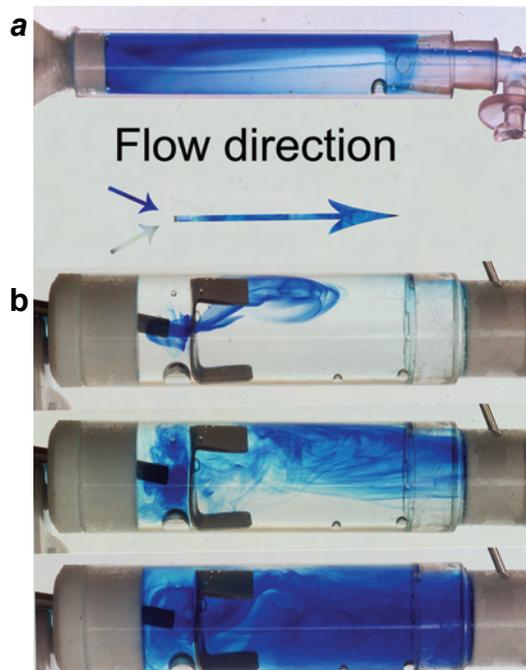
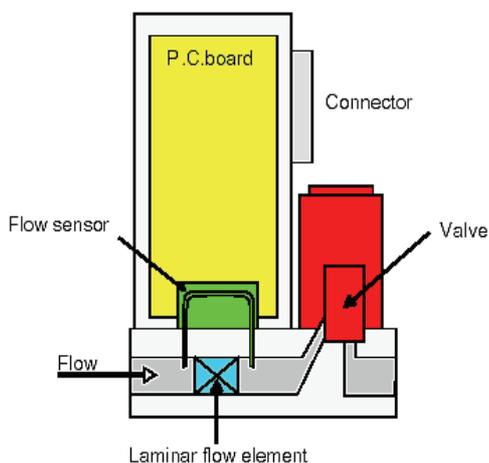


Fig 5



A mass-flow controller consists of a laminar flow element and a capillary bypass flow sensor with a heater between two thermosensitive resistors. A voltage proportional to the flow is produced and used to control an electromagnetic valve further “downstream” which controls the desired flow. The device will automatically compensate for variations in pressure and temperature

cylinder gas was diluted with oxygen at FiO_2 1.0 at 5 cmH₂O from 2 to 40 ppm. NO, NO₂ and the resulting oxygen levels were measured.

3.4 PAPER II

The delivery system described in (I) was used in a study of infants with RDS. We added a precision linear flow meter, SHO-rate (Brooks Instruments, Veenendaal, Holland), equipped with a pressure compensator for dosing NO. It was connected to a male quick connector QC4 (Swagelok, Solon, Ohio, USA). The Infant Flow Driver was used for fresh gas delivery and mixing. A sampling circuit incorporating a valve for constant pressure set at 30 cmH₂O was constructed to feed a NOxBOX and a paramagnetic O₂ analyzer (73), the OscarOxy (Datex-Ohmeda) in parallel. This allowed adjustments to provide the same FiO_2 that was delivered by the Infant Flow driver before dilution of inspired gas by iNO or placebo (N₂). CPAP pressure was manually adjusted as needed and kept constant with the help of the precision pressure monitor Digitron 2081P, (Digitron, Torquay, Devon UK).

Preterm infants (n=15) were studied in a blinded crossover design. The infants were randomly allocated to one of two sequences; iNO-Placebo or Placebo-iNO. Inclusion criteria were: typical signs of RDS, with a a/A PO₂ ratio ($PO_2 / ((95 \times FiO_2) - pCO_2)$) between 0.13 and 0.22, CPAP ≥ 4 cmH₂O, informed parental consent, weight ≥ 1000 g and GA > 27 w, lack of infectious symptoms, VOC and brain damage. All procedures followed rules for good clinical practice (GCP). Blood gases were analysed at a SWEDAC accredited laboratory.

Timeline

Experiments were started after a 15 min stabilisation period, during which flows, pressure and oxygen concentration were adjusted as follows: An open N₂ source was used to make room for the “would be” flow of the study gases to be added later. By means of a QC4 connector the gas supply could quickly be changed according to sequence. Study gas was administered – according to protocol - for 30 min each, with 15 min washout periods in between (Fig 6)

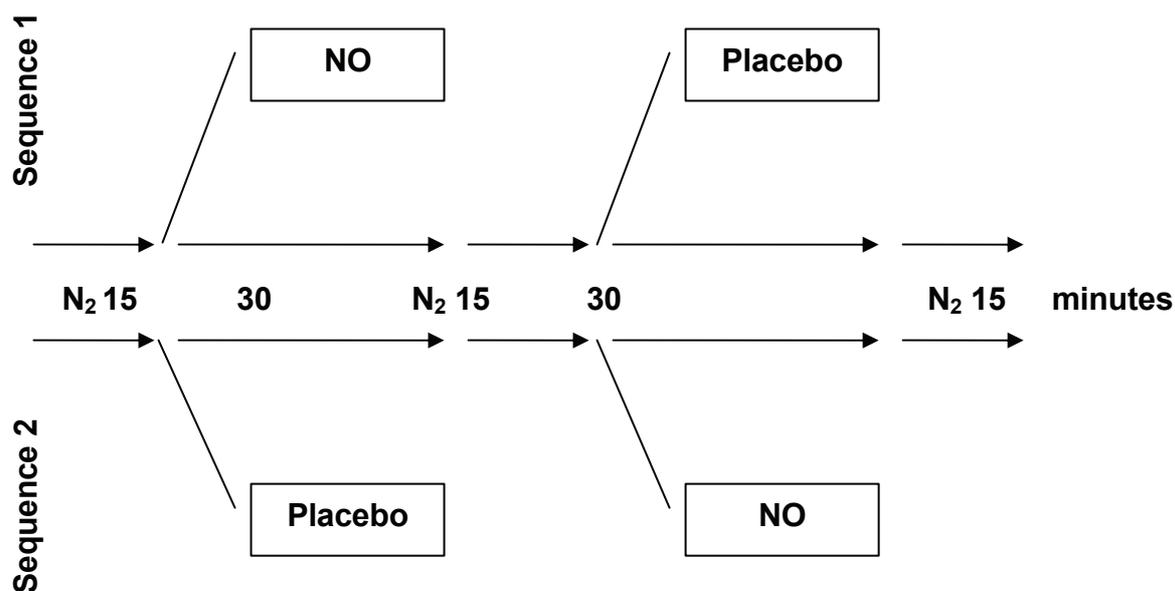


Fig 6, Timelines for gas administration

Patient monitoring was carried out by a paediatrician blinded to the actual gas sequence and followed a case report form (CRF). The M1095A monitoring system (Hewlett Packard, Boeblingen, Germany) was used to measure peripheral oxygen saturation (SPO₂) from the right hand, transcutaneous measurements of arterial oxygen tension (P_{tc}O₂), carbon dioxide tension (P_{tc}CO₂); with a probe fastened on the upper chest, heart rate was derived from a 3-point electrocardiogram. Invasive arterial pressure was obtained using the umbilical artery catheter. Blood gases were collected before, during and after each study gas.

3.5 PAPER III

We used the AC31M chemiluminescence analyser (Environnement S.A, Poissy, France) for measuring NO and NO_x in the ppb range and several NO_xBOX analysers for measuring NO and NO₂ in the ppm range.

Ppb calibration was carried out with a 1:20 dilution in nitrogen of NO 1.07 ppm in nitrogen (53,5) ppb and NO₂ 1.83 ppm in synthetic air (91,5 ppb) (Linde Gas, Höllriegelskreuth, Germany), using a mass flow controller device, the Nomius (Dansjö Medical AB) (fig 5) and Nitrogen 5.0 (AGA, Sweden) linearity was checked by stepwise reducing the dilution until pure gas was delivered.

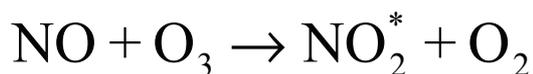
Ppm calibration: we used INOcal 45 ppm NO in nitrogen, and 10 ppm NO₂ in synthetic air (Scott Medical Products, Plumsteadville, Pennsylvania, USA)

For oxygen measurements the OscarOxy was used.

Data was collected in the form of analogue voltage signals from the different analysers. The signals were digitised via a PMCIA data acquisition card, DAQ card 6024E (National Instruments Corporation, Austin, Texas, USA) at a sampling frequency of 30 samples / channel / sec, collected via a custom written program using the Labview™ 7 software (National Instruments) and then processed and presented in Matlab™ (MathWorks, Natick, Massachusetts, USA). Statistics were performed in Statistica™ 7 (StatSoft, Inc. Tulsa, Oklahoma, USA)

chemiluminescence

A chemiluminescent reaction takes place between NO and ozone (O₃). This reaction is routinely used to determine either ozone (using excess NO) or NO (using excess O₃). The reaction is shown in the following equations:

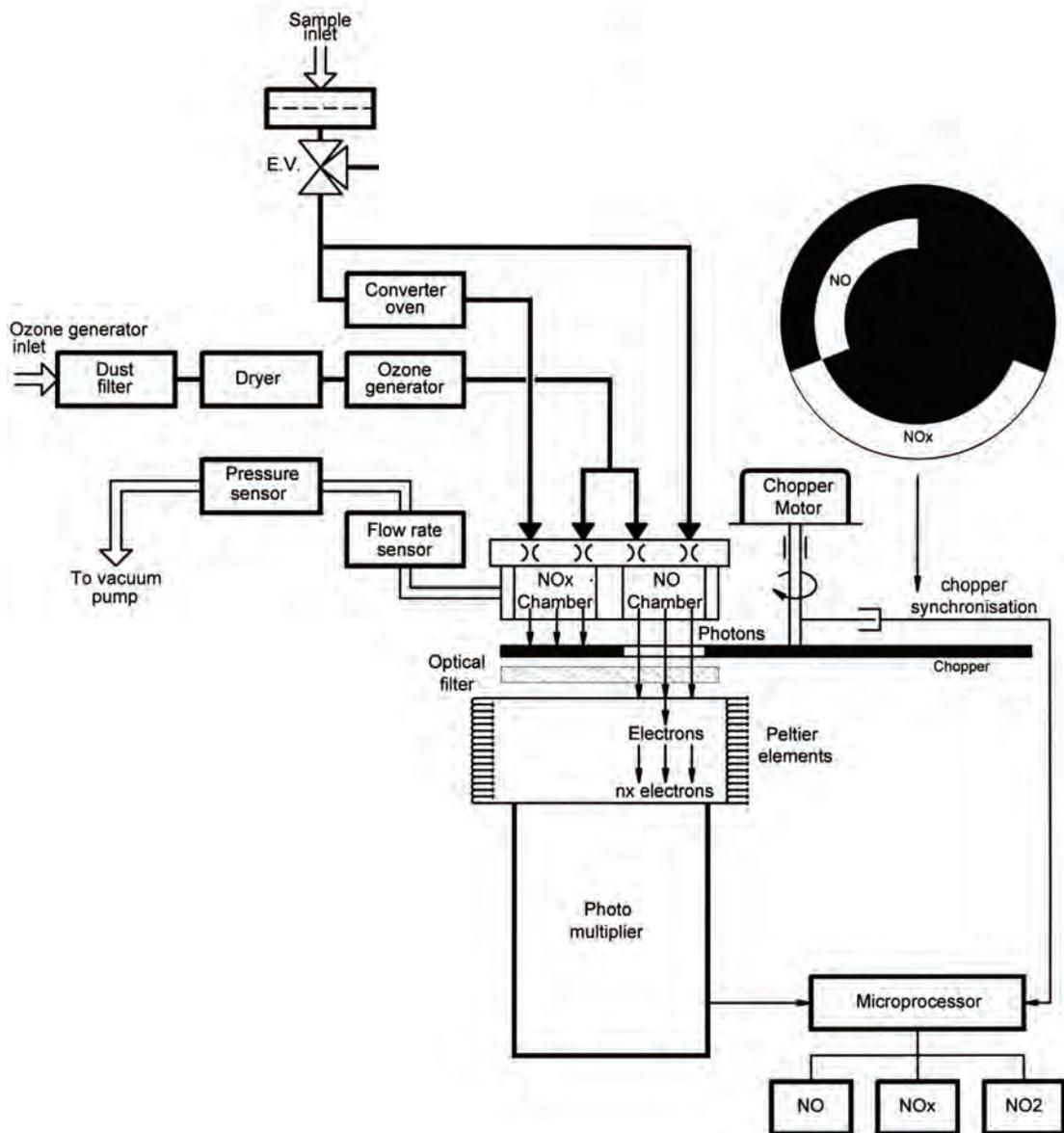


NO reacts with ozone to produce NO₂ in an excited state (denoted by the raised asterisk). Little of the excess energy involved in this process is released as heat; therefore, the reaction mixture and products do NOT incandesce to any significant degree. The reaction produces an excited state NO₂ which returns to a lower energy state by releasing photons of light: chemiluminescence. This electromagnetic radiation has a range of wavelengths; however, the emission is centred on 1200 nanometres (nm) and can be detected with a cooled red sensitive photo multiplier tube.

The conditional words in part are included in the last paragraph because there is actually two ways excited state NO₂ can de-excite. One is via photon emission (chemiluminescence); another is by losing energy through collisions with other particles. This collisional process becomes more and more significant as the amount of particles available for collisions increases at higher pressures. This is why most gas phase chemiluminescence reactions are performed at low pressures; this increases the amount of energy released via photon emission by decreasing the amount of collisional deactivation.

NO_2 can be measured as the difference between NO_x and NO . All NO_x is first reduced to NO . The efficiency of the converter has to be known or negative NO_2 readings can occur as NO_2 many times is insignificant compared to NO and NO_x

The AC31M (representation below) can measure both NO and NO_x by using a fast rotating disc known as a chopper (upper right) to alternately let the photomultiplier tube detect a signal from NO or NO_x reacting with ozone.



3.5.1 Simulated iNO CPAP treatment

Setting. A newly built ICU equipped with a displacement ventilation system at Danderyd University Hospital, Stockholm, Sweden. As a part of the construction procurement the ventilation system was recently tuned and all flows documented. The room of 126 m³ was ventilated at 470 m³ / h according to the protocol provided by the hospitals construction bureau (Dalkia Facilities Management AB, Stockholm, Sweden).

We used the custom designed delivery system described in (I) and (II).

NO gas. Medical grade gas from INO Therapeutics (Clinton, New Jersey, USA) was used. Cylinder concentration was 100 ppm NO in nitrogen.

Setup. We carried out 9 simulated treatments of iNO CPAP each lasting 90 minutes. We used a C100 incubator (AIR SHIELDS- HILL ROOM) with a volume of 150 l with the Infant flow™ nCPAP system inside to achieve accumulation of NO and NO₂. We used 10 ppm NO in 90 % O₂. The total flow was 8 l / min.

In order to sample from *three* separate sites in the room to the AC31M™ we constructed a switch, consisting of 3 micro solenoid valves no: VDW-13-5-G-1 (SMC Pneumatics corp., Tokyo, Japan) that were relay controlled so that only one was opened at a time and that time was controllable (Fig 7 a+b). The sampling sites were: inside the room ventilation inlet, inside the room ventilation outlet or a breathing zone sample: This position corresponded to having the face of a nurse / breathing zone 10 cm above the open right front hatch of the incubator during the care of the child.

Prior to starting the gas administration the room levels were measured for 15 minutes. The hatches of the incubator were kept closed until NO level in the incubator stabilized through passive leaks. This occurred after approximately 20 minutes. Thereafter, simulating treatment of the infant one hatch was opened in synchrony with the breathing zone measurement of the cycle. The hatch was opened three times for 1 minute at intervals of 6 minutes. All gases were shut off after incubator again had approached steady state to represent discontinuation of treatment. Analysis of NO/ NO₂ in room air continued for another 20 minutes



Fig 7 a+b, Relay controlled switch

3.5.2 One week background gas sampling

In order to provide data on the variations of the background over time we set up the AC31M™ to measure ventilation inlet levels of the same room described above over seven days starting on 5th of December 2004. No patient received iNO treatment in this room during that time.

3.5.3 Total release of gas from NO stock cylinders

An isolation room of 55 m³ was used, ventilation outlet measured as 14.4 m³ / min, which replaced the air 16 times per hour. NO and NO₂ was measured with both techniques centrally in the room, and in one corner to detect eventual uneven distribution and lingering gases. The measurement with chemiluminescence was performed with 50 seconds at each position whereas the fuel cells measured continuously. The sample frequency was 125 HZ. The process was monitored for 18 min until gas concentrations approached baseline.

A 10 litre gas cylinder pressurised to 150 bar with iNO max™, NO 400 ppm (iNO Therapeutics, Clinton, New Jersey) without gas regulator attached anchored to the wall with chains. The valve was fully opened and the cylinder allowed to empty completely during approximately 3 min. The resulting gas concentration in the room was recorded from outside the room.

The process was repeated with a 20 litre gas cylinder pressurised to 150 bar with NO 1000 ppm of medical quality with a NO₂ content < 5 ppm (AGA Speciality gases, Lidingö, Sweden). Emptying time was 5 min.

3.6 PAPER IV

Sedated 1-2 month old healthy piglets (n=33, Swedish landrace) were studied for 4 hours during exposure to 100 ppm NO in air versus control groups and a group exposed to 10 ppm NO₂ in air (Fig 8a). An adjustable mask (Fig 8b) was connected to a jet entrainment device, the Multi-Vent[®] (Hudson RCI, Temecula, CA, USA). Medical grade 1000 ppm NO in nitrogen (AGA Healthcare) used to drive the mask, at a mixture of 1:10 between NO and room air. To administer NO₂, the Multi-Vent was adjusted to give an exact mixture of 1:3 (NO₂: air) utilizing 30 ppm NO₂ (AGA) in air as driving gas.

The CS/3 (Datex-Ohmeda, Finland) monitored that the gas flow into the mask was sufficient to avoid rebreathing or inhalation of room air at inspiration by using paramagnetic O₂ and infrared CO₂ analysis. The NO gas flow was controlled using a Sho-Rate[™] 15C precision logarithmic rotameter (Brooks Instrument). The gas mixture was monitored with a NO_xBOX[™] (Bedfont Scientific) and a Nomius C (DanSjö). Both monitors were calibrated with a calibration gas containing NO 85.3 ppm / NO₂ 6.8 ppm ± 1 % Lownox (AGA Speciality Gases, Lidingö, Sweden).

Arterial blood gases were measured hourly in an ABL 5 (Radiometer), with tonometric correction for pig blood.

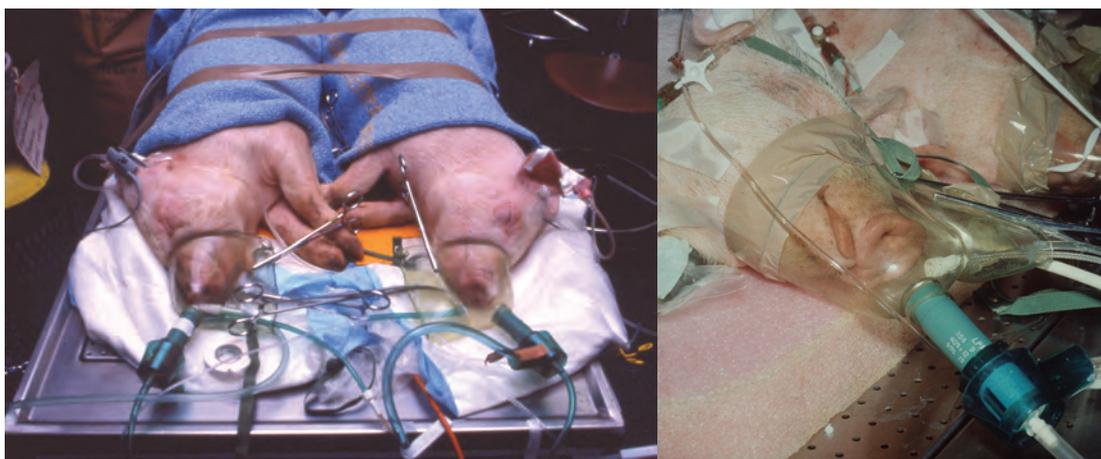


Fig 8 a + b, The piglets were anaesthetised and exposed to NO or NO₂, two at the time, on a table with good ventilation in order to reduce exposure of the staff to the gas mixtures. Concentrations of oxygen, carbon dioxide, NO and NO₂ were measured at the snout. The piglets were covered with a quilt and had electrical heating blankets.

Processing of surfactant

The lungs were lavaged with saline 0.9%, via an endotracheal tube inserted post mortem in the airways. The pooled bronchoalveolar lavage fluid from each animal was first centrifuged at 100g for 15 minutes to remove cell debris, then at 5,000g and 4° C for 2 hours. The pellet was resuspended in saline 0.9%, 1/10 of sample volume was extracted with chloroform : methanol (2 : 1) and phospholipid content was determined (7). The remaining 9 / 10 of the sample volume of natural surfactant were then standardized at 2 mg/ml by dilution with saline 0.9%. Dynamic bubble size surface tension, of the samples was measured in a pulsating bubble surfactometer (Electronetics Corporation, Amherst, NY, USA) at 37 °C during 50 % cyclic surface compression at a rate of 38 cycles / min for 5 minutes. Minimum surface tension values (γ min) for the 5th cycle, 1, 2 and 5 minutes were determined.

Animals were divided into two exposure groups. One group (n=12) received 100 ppm NO in air for 4 hours, the other (n=6) 10 ppm NO₂ in air for 4 hours. Two groups were used as control. Animals that were anaesthetised and directly killed were lavaged and served as main control (n=11). A model control group (n=4) was added to investigate if the anaesthesia in itself altered surfactant function. They were allowed to breathe air for 4 h, then killed and lavaged.

Statistics

Non parametric statistical procedures were used throughout this paper. The Mann-Whitney U-test was employed for single statistical comparison of independent groups of samples and the Kruskal-Wallis analysis with Dunn's posthoc test for multiple comparisons of independent groups of samples. A p-value of less than 0.05 was considered significant.

The statistical software used was GraphPAD InStat for Windows (version 3.05, GraphPad Software Inc., San Diego, CA, USA).

3.7 PAPER V

Sixteen male Chinese piglets were studied. The SC 9000 monitor (Siemens, Solna, Sweden) was used to record vital life signs. A 4F Swan–Ganz catheter was inserted into external jugular vein to measure central pressures.

NO 1000 ppm stock gas (Shanghai BOC) was used. It contained < 1% NO₂ relative to NO as determined with a chemiluminescent analyser (Sievers NOA280, Boulder, CO). The NO gas was piped to the inspiratory limb at a constant flow regulated by a mass flow controller (143). NO and NO₂ in the ventilator circuit were monitored continuously with a NOxBOX.

After instrumentation, the animals were randomised to groups receiving iNO (NO group) or no iNO (Control) The Control group received CPAP and pressure support ventilation (PSV) for 24 h (n= 4) or 48 h (n= 4); the NO group received the same assisted ventilation plus iNO diluted to 40 ppm for 24 h (n= 4) or 48 h (n= 4).

Every third hour lung mechanics were recorded with a pneumotachograph GM 250 Navigator (Newport Medical Instrument, Newport Beach, CA) and arterial blood gases collected.

Methaemoglobin (MetHb), nitrite and nitrate in blood serum were measured at 0, 3, 6, 12, 24, 36 and 48 h. Values are expressed as mmol / l. The animal was sacrificed and the right middle lung lobe was used for measurement of wet-to-dry weight ratio (W/D) (143). The remaining part of the right lung was lavaged with 0.9% NaCl at 15 ml/kg body weight. The left lung was perfused for 30 min via the left branch of the pulmonary artery with 4% formaldehyde at a pressure of 65 cm H₂O (143).

Amounts of disaturated phosphatidylcholine (DSPC) and total phospholipids (TPL) in bronchial alveolar lavage fluid (BALF) were determined according to Mason et al. (68) and Bartlett (7). Total proteins (TP) in BALF were measured according to Lowry et al, corrected by total volume of BALF and body weight. Measurements of surface tension of phospholipids from BALF were carried out with a pulsating bubble surfactometer (PBS, Electronetics, Buffalo, NY) set at 25 cycle/min. After 5 min of sonication the samples were adjusted to a phospholipid concentration of 5 mg/ml. Minimum and maximum surface tension at 5 min (γ_{min} and γ_{max}) were obtained at minimum and maximum bubble size, averaged from four determinations for each sample, and expressed as

milli Newton per meter (mN/m). Five representative lung tissue blocks from the left lung were embedded in paraffin, and sections stained with haematoxylin and eosin were examined by light microscopy. In a blinded manner, one of the authors (Zhu) carried out microscopy of the pig lungs. Lung injury was scored for oedema, haemorrhage and inflammatory cell infiltration. A score represented the severity: 0 for no or very minor to 4 for widespread and most prominent (142). Lung expansion was quantified and expressed as volume density (Vv) of aerated alveolar spaces, using total parenchyma as reference volume (26). Fifty fields of each animal lungs were examined (magnification x 300), and field-to-field variability was determined by calculating the coefficient of variation of Vv (CV(Vv)). A low value for CV(Vv) indicates homogeneity of alveolar aeration.

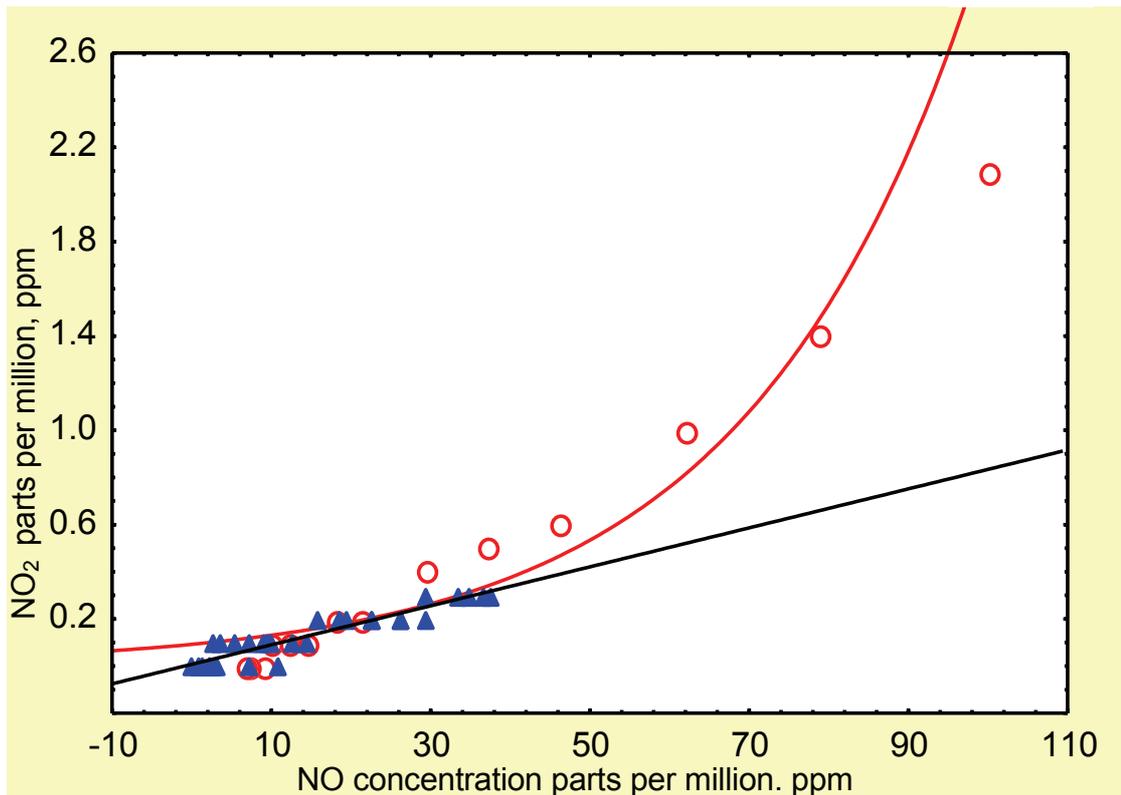
Statistical analysis

Continuous parametric data were subjected to Wilcoxon–Mann–Whitney test. Within group difference was examined by Wilcoxon signed rank test. For the lung injury score, a Kruskal–Wallis test was used followed by Wilcoxon–Mann–Whitney test for difference between the two groups. A p-value < 0.05 was regarded as statistically significant.

4 RESULTS

4.1 PAPER I

In the 50-ppm stock gas experiment, the NO_2 peak value was 0.3 ± 0.0 ppm at 36.6 ± 1.0 ppm NO. Using 1000 ppm gas with an FiO_2 of 0.80 ± 0.02 the maximum NO_2 values were 0.5 ± 0.1 at 36.7 ± 0.8 ppm and the NO concentration at which NO_2 values above 2 ppm were first observed was 95 ppm



(Fig 9) Resulting NO_2 concentrations for dilutions of 50 ppm (**blue** Triangles) and 1000 ppm (open **red** circle). NO stock gas. The line fit representing the 50 ppm gas is shown as a black line. The curve fit for 1000 ppm. is shown as a **red** curve. More NO_2 would have been formed if 100% oxygen had been used to dilute the 1000 ppm gas instead of keeping a 80% final oxygen concentration further separating the line and curve. Mixtures above 40 ppm using the 50 ppm cylinder would have resulted in hypoxic O_2 levels.

4.2 PAPER II

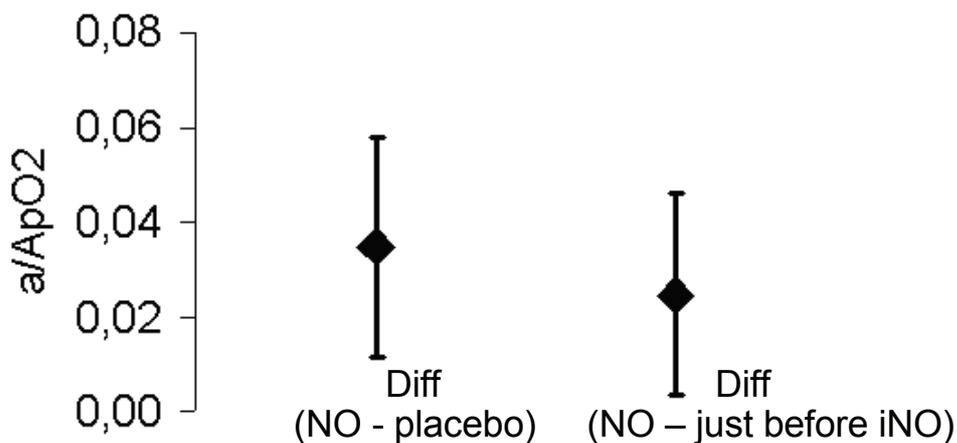
For the whole group alveolar to arterial O₂ tension difference (aA PO₂) was 0.19 ± 0.06 before study gases and under placebo but increased under iNO treatment to 0.22 ± 0.05 (+20 %) (p = 0.006, 95 %), (r:II:1) and (r:II:2). SPO₂ was 96 ± 3% under iNO and 93 ± % during placebo (p < 0.05), (r:II:2a). Respiratory rate, partial pressure of CO₂ in blood (PaCO₂), arterial pressure and heart rate was unaffected during exposure to iNO (r:II:2). NO₂ was 0.3 ± 0.1 ppm at the delivered NO concentration of 9.4 ± 1.7 ppm. CPAP was 4.4 ± 0.4 cm H₂O. The increase in aA PO₂ during iNO was more pronounced in infants with a gestational age < 34 wk (n = 9, 95 % CI for aA PO₂ – difference: 0.006 - 0.08 compared to more mature infants (n = 6, 95 % CI for aA PO₂ – difference: - 0.002 - 0.06. Methaemoglobin values did not change.

All patients recovered completely and no patient required oxygen at 30 days.

(Tab) 1 Outcome data (mean ± SD)

	Baseline	Placebo	iNO 10 ppm	P value iNO vs Placebo
aA PO₂	0.19 ± 0.06	0.19 ± 0.05	0.22 ± 0.05	0.006
Respiratory rate (RR / min)	63.0 ± 16	59 ± 16	68 ± 20	n.s.
PaCO₂ (kPa)	7.03 ± 1.19	6.97 ± 1.24	6.92 ± 1.13	n.s.

(Fig 10) Diff (a/APO₂) 95% conf



4.3 PAPER III

Occupational exposure

After 20 min the NO level in the incubator was 3.4 ± 0.6 ppm and fell to 1.4 ± 0.4 ppm when the hatch had been open for one minute. The level of NO₂ inside was 0.24 ± 0.05 ppm. The resulting NO/NO₂ maximum levels of mean values in the breathing zone 10 cm from the hatch were 18 ± 7.5 ppb NO and 25 ± 6 ppb NO₂ respectively. The latter value was detected 6 min after the maximal NO value. The breathing zone levels did not become significant ($p < 0.05$, Mann-Whitney U-test) until the end of the third hatch opening.

Due to the weather, contribution from the traffic in the area during the study, gave low stable inlet values for NO 3.2 ± 2.4 ppb, and NO₂ at 11.0 ± 10.4 ppb.

Subtracting the room inlet values at each point the maximal additional contribution of NO and NO₂ from the iNO-CPAP to the breathing zone values was 14.8 ± 5.5 ppb for NO and 14.7 ± 4.9 ppb for NO₂. The subtracted values for the room levels equalled an addition of 4.2 ± 3.4 ppb for NO and 9.6 ± 5.2 ppb for NO₂.

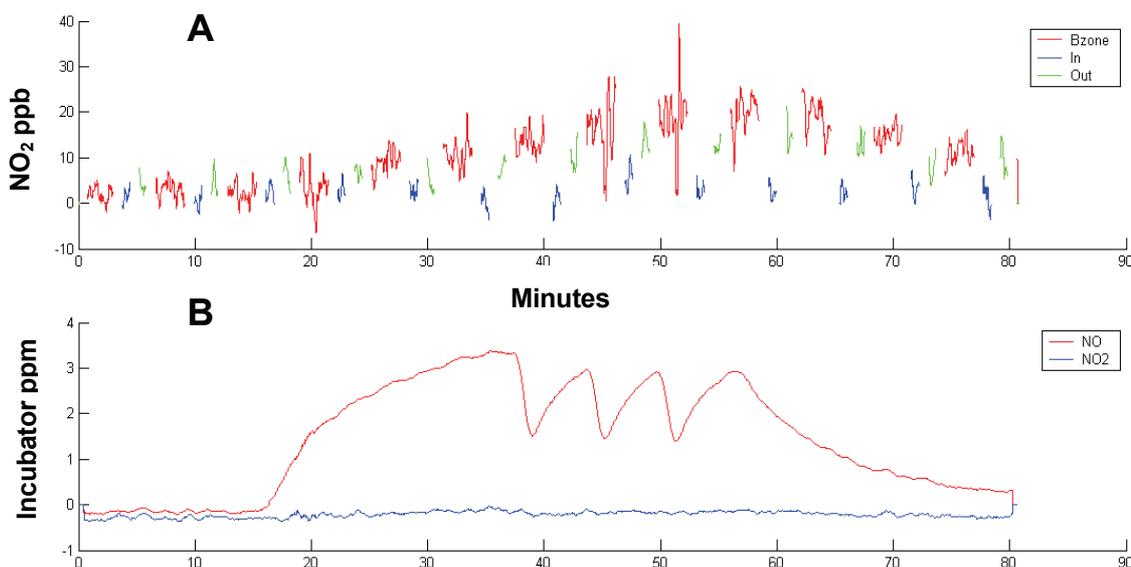
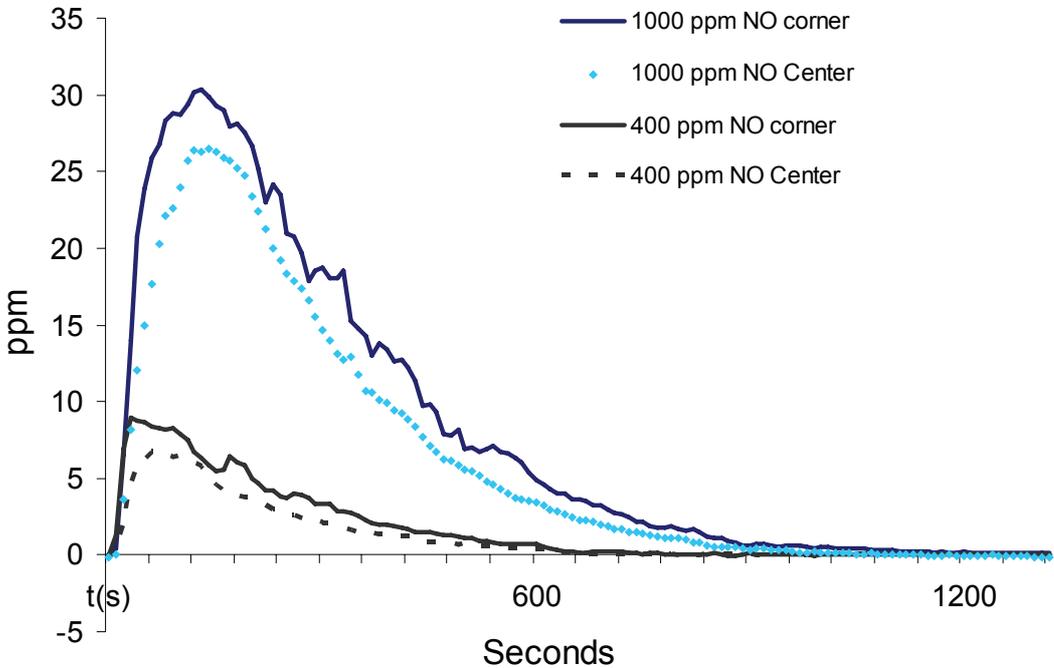
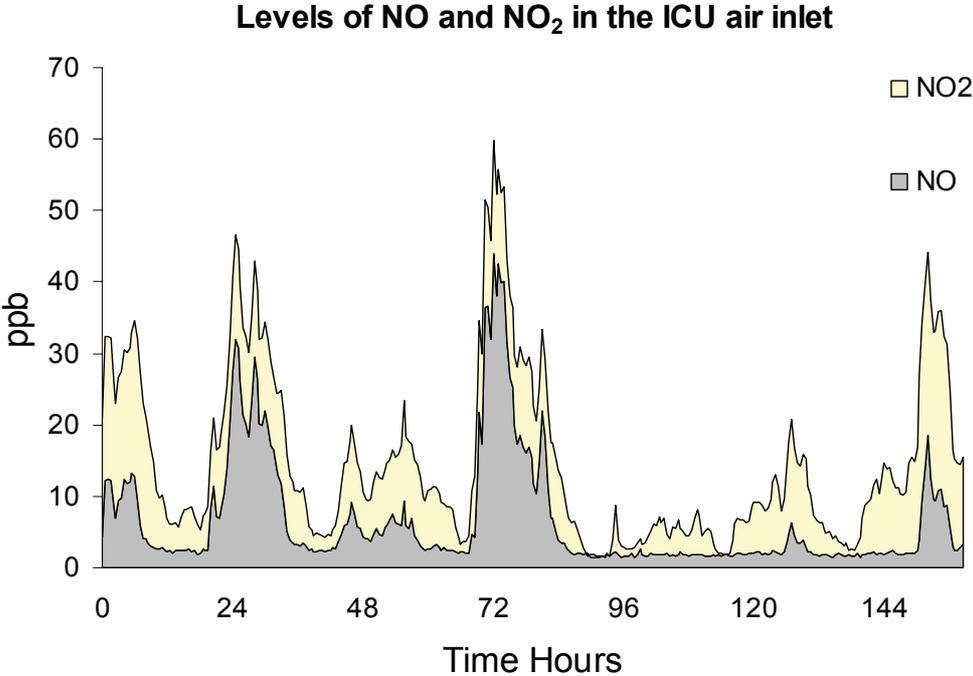


Fig 11A: Sequential NO curves from three measuring sites: breathing zone (red), room inlet (blue) and room outlet (green) scale ppb. Time for each cycle: 6 minutes.

Fig 11B: NO (upper curve, red) and NO₂ (lower curve, blue) concentrations in the incubator measured by Bedfont NOxBOX fuel cells. iNO administration (10 ppm) was performed between 15 and 60 minutes. The three notches in the NO curve of the incubator represent hatch openings.

(Fig 12) At a later recording of weekly background levels gave min / max of 1.5 - 44 ppb for NO and 0 -26 ppb for NO₂ (below).NO_x is represented by adding NO+NO₂.



(Fig 13) Mean NO in the room after release of 1000 ppm and 400 ppm cylinders corresponding to 3000 and 1500 litres of gas respectively.

A simulation of accidental emptying of cylinders

The total emptying of the cylinder equal to 1500 L. of NO 400 ppm produced a maximum value of 8 ppm NO and 0.2 ppm NO₂ whereas the release of the cylinder equal to 3000 L. of NO 1000 ppm produced a maximum level of 30 ppm NO and 0.8 ppm NO₂, the NO₂ peak level appearing 80 seconds after the peak for NO. T_{1/2} was 142-149 seconds for NO 400-1000 ppm for the corner sampling point and 122-116 seconds for the sampling point central in the room. NO₂ T_{1/2} values could only be calculated with certainty for the 1000 ppm NO release experiment and was 200 seconds for the corner sampling point and 145 for the middle of the room. Time to return to background levels were 15 and 20 minutes respectively.

4.4 PAPER IV

All animals completed the intended study protocol and displayed stable vital signs. NO levels were kept stable at 100 ± 1.8 ppm. The resulting FiO₂ was 0.19, due to dilution of air with N₂ that served as a carrier gas. Levels of formed NO₂ were 1.0 ± 0.3 ppm.

Exposure to piglets of iNO for 4 hours

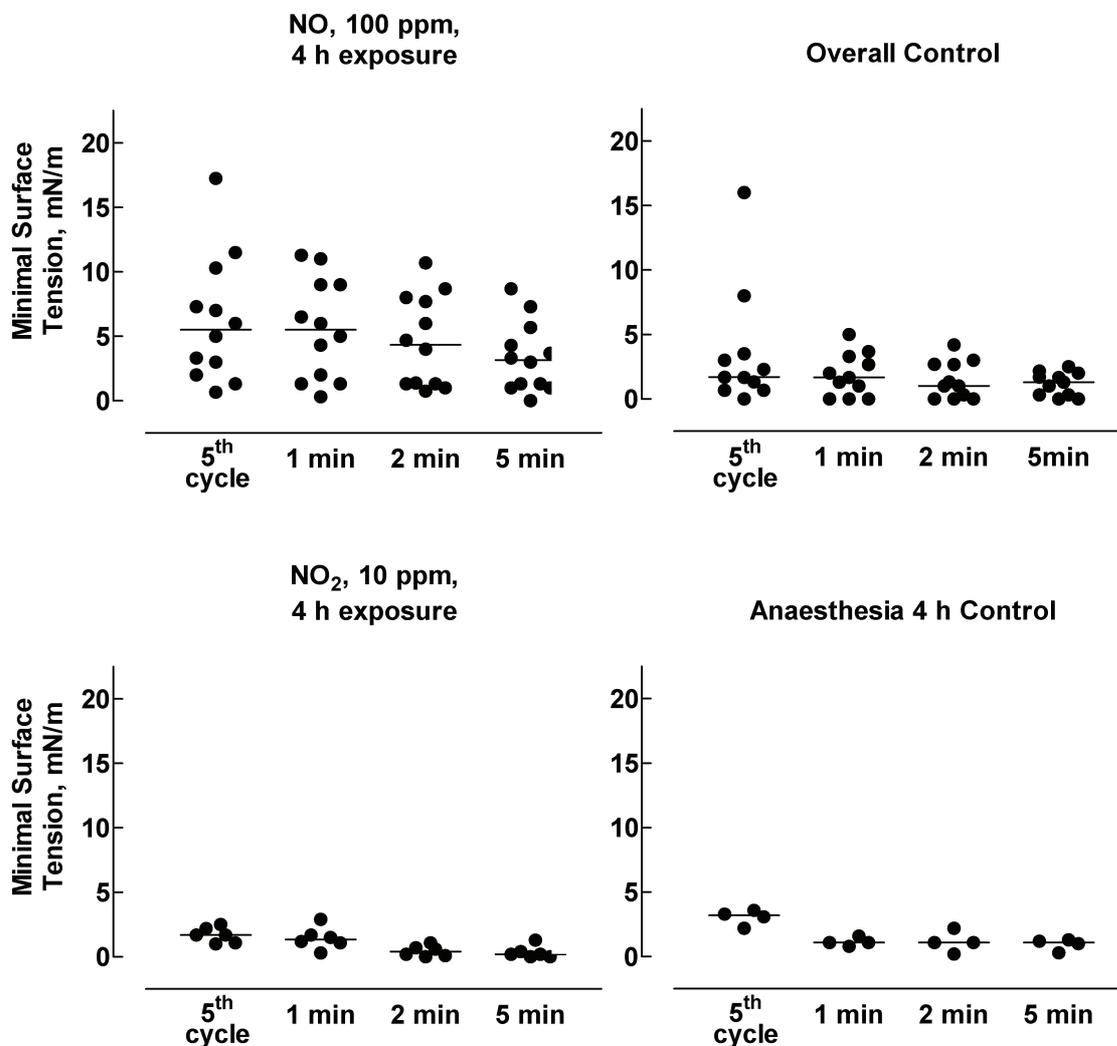
Exposure of iNO caused a slight but statistically significant impairment of the surface tension reducing capacity of the surfactant. After 1 minute, γ min was 5.6 ± 3.9 mN/m in the iNO group compared to 1.2 ± 0.3 mN / m in the 4h anaesthesia control group ($p < 0.05$), and 1.9 ± 1.7 in the control. At 2 and 5 minutes the slight increase in γ min in the iNO group, remained significant compared to control.

Exposure to piglets of NO₂ for 4 hours

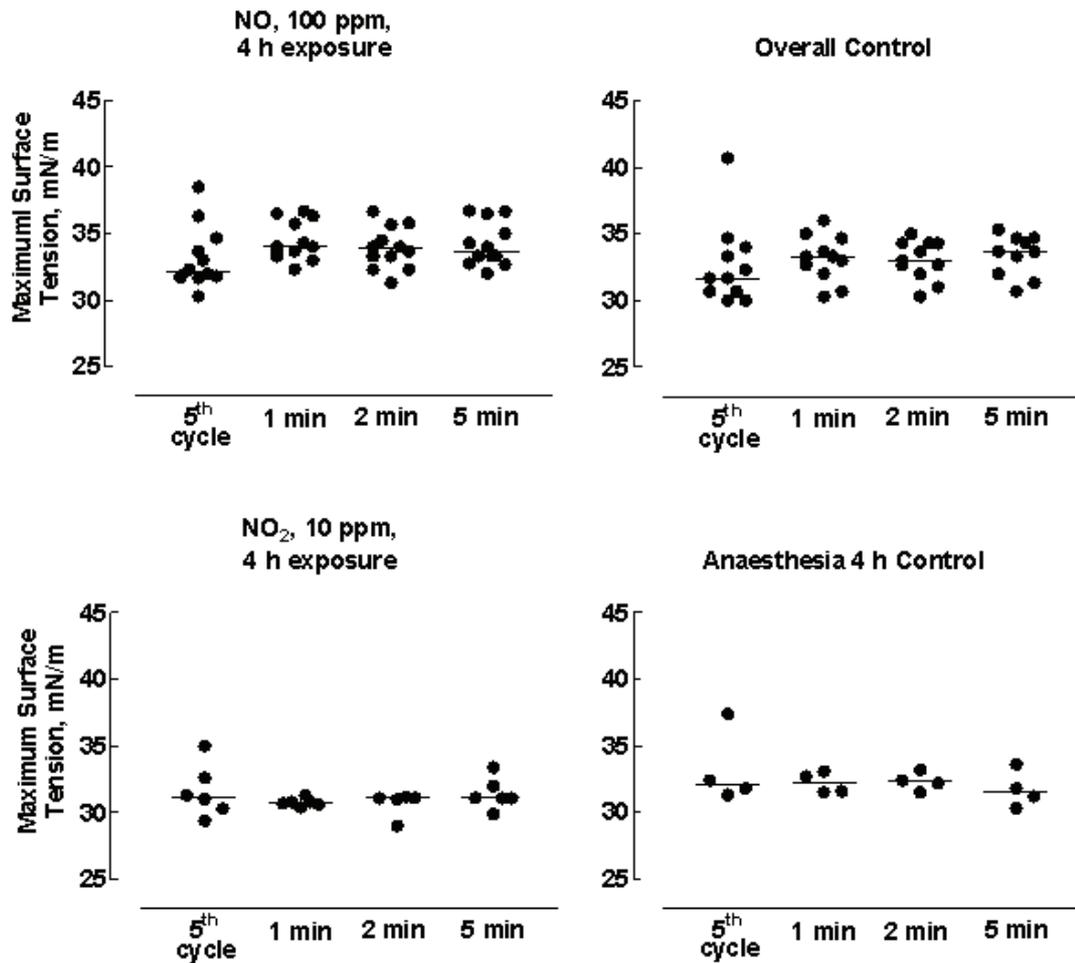
NO₂ levels were kept stable at 9.8 ± 0.4 ppm. Contrary to our expectations were there no clinical signs of airway irritability such as cough or wheezing, nor

any desaturation. No elevation of γ min was found in surfactant from piglets exposed to 10 ppm NO₂ for 4 h against both controls.

(Fig 14 Below): Surface tension measurements from a pulsating bubble surfactometer for **minimum** surface tension. Natural surfactant at 2mg/ml.



(Fig 15 Below): Surface tension measurements from pulsating bubble surfactometer for **maximum** surface tension. Natural surfactant at 2mg/ml.



Maximal surface tension gave a less consistent result. NO₂ had lower maximal surface tension than NO and overall control at 1, 2, and 5 minutes and vs. anaesthesia 4 h control at 1 and 2 minutes ($p < 0.05$)

4h control animals (anaesthesia only) had lower maximal surface tension than overall control (directly killed) at 5 minutes of pulsations.

Anaesthesia 4 h control had lower surface tension than NO exposed animals at 5min of pulsations.

4.5 PAPER V

In the NO group methemoglobinemia occurred as MetHb increased steadily from $0.88 \pm 0.17\%$ at baseline to the highest mean level reaching 5 % at 24 h consequently serum nitrite/nitrate increased significantly in 3 h of NO

inhalation and reached 263 ± 114 mmol/L at 6 h. At 48 h nitrite/nitrate were 352 ± 90 mmol/L in the NO and 88 ± 38 mmol/L in the control group.

There were no statistical differences in vital statistics. All the animals survived the intended experimental period. The concentration of iNO was kept at 40 ppm and NO₂ at 1 ppm or less as measured with a NOxBOX fuel cell.

Mean levels of tidal volume and minute volume ventilation were maintained for both groups at 5–8 ml/kg and 0.3 L/min kg, respectively, during the ventilation period (24–48 h), and peak insufflation pressure of PSV at 10–12 cm H₂O at time 0 h to 15–20 cm H₂O at 48 h, with no significant differences between the two groups. The mean level of PaCO₂ was kept at 30–45 mmHg, along with the mean arterial pH, standard bicarbonate (SBC) and base excess (BE) in the ranges of 7.40–7.50, 21–30 mEq/L and 21.2–5.0 mEq/L, respectively. Partial pressure of oxygen in blood (PaO₂) was kept at 60–90 mmHg when FiO₂ was increased to 0.3 at 12 h in the NO and at 24 h in the Control group.

A maximum FiO₂ level of 0.4 and 0.34 was required for the NO and Control group, respectively. PaO₂ / FiO₂ in both groups trended to decline over time to, 300 mmHg (results 1). Using paired two sided students T test the control group displayed a significant worsening of PaO₂ / FiO₂ ratio from 0 to 24 hrs ($p=0.029$) whereas the drop in the iNO exposed groups did not become significant at 24 hrs.

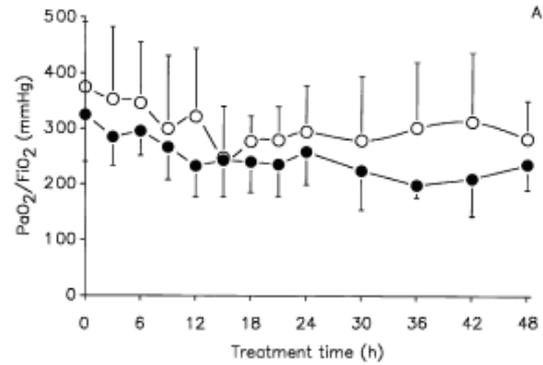
Values for 48 hours vs. the initial values were not calculated due to group size. The values of dynamic compliance (C_{dyn}) and resistance of the respiratory system (R_{rs}) of the two groups were stable during the experiment. The phospholipids from BALF had minimum and maximum surface tension around 10 and 40 mN/m, respectively, suggesting mild influence on biophysical properties, but not related to iNO.

White cell counts WBC from BALF in both groups were not significantly different. No significant difference in W/D ratios was found across the subgroups, but the average values of the NO group were lower than that of the Control group at 24 and 48 h.

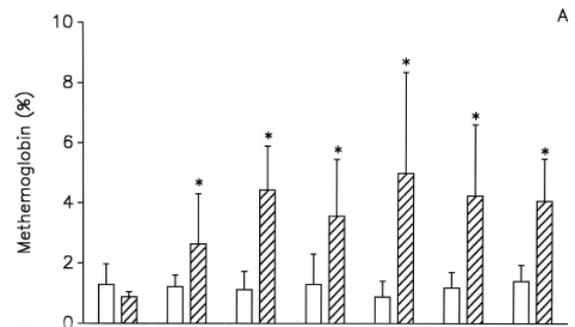
Inflammation was found in the lungs from both groups, which was characterized by mild diffusive oedema and neutrophil infiltration in the interstitium and alveolar space in some areas of the lungs. Mild or moderate atelectasis was also found in the lungs. Although most of the lung tissue had an almost normal structure, the lung injury scores and V_v were similar between

the two groups at 24 h. More inflammatory cells were found in C48 subgroup compared to the C24 subgroup ($P < 0.05$).

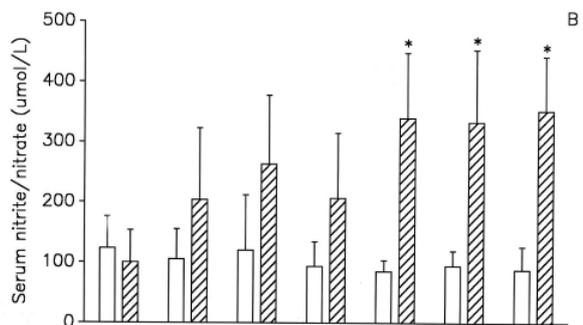
(Fig 16) Arterial oxygen tension to inspired oxygen fraction (PaO_2/FiO_2)



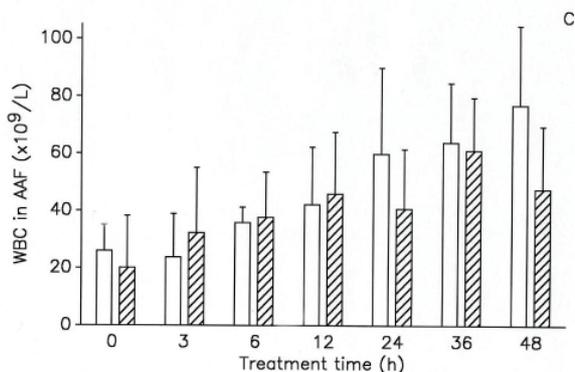
(Fig 17) (A) There was a significant increase in methaemoglobin after 3 hour and onwards. * $p < 0.05$ vs. control group



(Fig 17) (B) The elevations in serum nitrite / nitrate only become significantly increased after 24 hrs and onwards. * $p < 0.05$ vs. control group



(Fig 17) (C) There was a tendency toward higher white blood cell counts in Airway aspirates with numerically higher values for the controls. This trend did not become significant.



(Tab 2) Summary of characteristics, values given as mean and SD, * p < 0.05 vs C24.

Group	C24	C48	NO24	NO48
Oedema	0.75 ± 0.34	1.90 ± 0.68	0.75 ± 0.90	0.85 ± 0.25
Haemorrhage	0.30 ± 0.20	0.45 ± 0.53	0.15 ± 0.30	0
Inflammation	0.15 ± 0.19	1.30 ± 0.35*	0.45 ± 0.30	0.80 ± 0.37
WBC (x 10⁹/L)	63 ± 40	195 ± 159	65 ± 40	154 ± 72
W/D	6.1 ± 0.4	6.2 ± 0.5	5.5 ± 0.5	5.7 ± 0.5
Y_{min}	10.2 ± 3.7	14.8 ± 5.9	9.8 ± 1.6	11.2 ± 4.8
Y_{max}	43.3±6.0	41.5±7.7	43.1±3.1	36.5±2.6

5 DISCUSSION

General

The current indication for iNO is as a late rescue intervention in cases of persistent foetal circulation (pulmonary hypertension) with the purpose to reduce pulmonary vascular resistance as well as improve oxygenation (65). An underlying theme in this thesis is to use iNO instead as an early intervention in respiratory distress syndrome (RDS). This necessitated the development and testing of a delivery system for iNO to non-intubated neonates, at a stage of illness when MV was not considered necessary. Earlier work indicate that lung recruitment is a prerequisite (89) for the oxygenation response brought about by iNO. Both sides of the shunt equation Q_s/Q_t , (a ratio of non-ventilated alveoli to perfused pulmonary capillaries) need to be addressed to improve oxygenation, making a combination with CPAP a logical choice. With this approach subsequent respiratory failure might be possible to avoid, making iNO a semi-prophylactic clinical treatment option in RDS. Support for this earlier exposure to iNO can be found in emerging experimental evidence on how endogenously formed NO is involved in normal lung development (116). In addition a dysfunction of NO-mediated signalling can be shown to play a role in the development of BPD (71; 119).

More liberal clinical use of iNO implies emphasis on systematic elimination of safety concerns.

A separate discussion accompanies each of the individual papers of this dissertation.

Discussion of methods

I. Very low levels of NO_2 were measured with fuel cells in inhaled gas at the detection limit of this method. Chemiluminescence could have been used instead but has problems to correctly measure NO_2 in the presence of higher levels of NO. NO_2 is calculated as the difference between NO_x and NO and is dependent on low errors in these measurements, efficiency of the converter,

water content in the gas etc, and negative NO₂ readings can occur. However higher levels of NO₂ had they occurred would easily have rendered themselves to be measured with the fuel cells. A formula that used changes in FiO₂ would have been a useful clinical tool to double check NO concentrations. Using 50 ppm stock gas clinically could both limit final oxygen concentration as well as leading to too frequent changes of gas cylinders unless very low levels of NO was the target. To overcome these difficulties 100 ppm gas was used in paper II while still allowing for a simplified administration system and keeping NO₂ formation low.

II Already from the onset we realised that the study design raised an ethical aspect since the administration of surfactant to these infants with stable mild to moderate RDS could be delayed by slightly less than two hours. Thus, we discussed whether it would be ethically acceptable to allow such a time delay purely for the purpose of a scientific study. Since we believed that the time delay was of limited duration and that it would be more difficult to detect any improvement of oxygenation after the administration of surfactant, it was decided to adopt and accept this study design.

Power calculations suggested that 22 subjects acting as their own controls had to be included versus 120 using separate placebo and active drug groups. The use of a crossover design enabled us to expose a lower number of infants to iNO. We felt that by creating this research tool and describing its characteristics, larger studies would be made possible. As the response to iNO in spontaneously breathing infants was unknown, a prudent approach would, thus be to limit the number of subjects and exposure time in the first study and to run it as a phase II pilot rather than to expose a larger number for longer time as a phase III study..

During the review process of this paper the ethical dimension of the study design was further discussed by assessors and the editor. However a number of factors support our initial stance regarding this matter. Firstly, the local ethical committee as well as Medical Products Agency (MPA) have accepted our discussion on the ethical issue and did, thus, with only small alterations approve of the study design. Secondly, only four out of fifteen infants received surfactant after the study and three of them several hours after end of study exposure, showing that the normal clinical situation did in fact cause substantial

delays, longer than the study itself. Furthermore, the satisfactory final outcome of the infants, with all infants recovering without complications, provides a final reassurance that no ethical trespass can be said to have occurred in this instance.

III. A possible limitation to the study is the *in vitro* model. Do the results of the study reflect a normal clinical situation? We believe that we have mimicked a worst case care situation at a treatment dose of 10 ppm iNO with gas released *inside* the incubator. The level of NO and NO₂ we measured from one child during actual treatment with iNO-nCPAP was in fact slightly lower than the values generated in the simulation suggesting that iNO to a large extent was absorbed when the same dose was given to a infant. If anything the study overestimates resulting NO - NO₂ levels.

It is also an interesting observation that the inlet air due to the pollution of the ambient environment on certain days clearly exceeds the NO - NO₂ levels generated by 10 ppm iNO-nCPAP treatment.

The choice of switched sampling introduced another limitation. The sequenced sampling from several sites, gave us the possibility to distinguish between different origins of nitric oxides were other authors (77; 87) have been unable to find a significant contribution from patient exposures to iNO. However if the sampling was switched to another site a peak could pass unnoticed. We made allowance for this in the protocol and only opened the hatch to incubator when the sampling was in the breathing zone.

IV. In this study the present-day clinical relevance of studying a high dose of 100 ppm iNO can be put in question. We had previously used this dose and time in several laboratory experiments exposing the surfactant proteins SP-B and SP-C in a dry-film preparation to NO in nitrogen and finding depalmitoylation of SP-C (57). Since this study was designed at a time when at least some authorities in the field published and advocated the use of 80 ppm iNO (95) we decided that a dose of 100 ppm could be considered of interest and would also result in a greater stress to the lung compared to doses of ≤ 80 ppm. A second issue is the short duration of the exposure (4 h). This was chosen partly because of the previous experiment and partly on practical grounds since we were aware of the difficulties in keeping anaesthetised

piglets breathing spontaneously for longer periods. We wanted to avoid the use of MV, as the possible changes in surfactant function we searched for might be so discrete that they would be impossible to distinguish from early lung damage caused by MV. Our concern was substantiated by data from paper V, in which piglets receiving minimal ventilatory support displayed a slow deterioration of gas exchange and at study end showed mild inflammatory changes in lung histology. We speculated that a larger dose would thus mimic longer exposure: the product would be “ppm-hours” as used by others (69).

The design of paper IV did not allow us to determine the effects on surfactant if lower levels of iNO or prolonged periods of exposure are used. It should also be noted that the piglets participating in paper IV had no lung damage induced. It is reasonable to assume that surfactant of lungs with some degree of lung damage might respond differently. Although the findings of the study cannot be taken as evidence that iNO at lower dosing are safe, it must be remembered that the negative effects of iNO on surfactant function in paper IV was only of mild-moderate nature. This observation is also in agreement with the now quite large clinical experience of the use of ≤ 20 ppm NO in severely ill neonates. In this population clinical use of iNO for a few days has not been associated with reports of negative effects on surfactant function.

V. When gas exchange data from the experiment were analysed and pooled, a slow significant deterioration in $\text{PaO}_2/\text{FIO}_2$ ratio over time was observed. In other words, the experiment evolved in similar to a slow lung injury model. We had aimed at providing minimal ventilatory support, and started with a PEEP of 3 cmH_2O and PSV, since we were dealing with healthy animals. No attempt to find an adequate PEEP level was done. In the light of the crude pressure trigger function and the few controls available to shape the PSV pressure curve on the ventilator Siemens 900C, one explanation could be that the inspiratory effort created a negative pressure that resulted in a discrete alveolar collapse on each breath before triggering occurred, thus creating a VILI situation.

The administration method of nitric oxide was of the constant flow type, regulated by a mass flow driver and supplied to the inspiratory limb of the ventilator. This had two consequences: 1) proportional dilution of inspired oxygen with nitrogen (carrier gas for NO), resulting in a lowered oxygen concentration in the first part of the breath as evidenced by the need for

additional oxygen in the NO group; 2) giving a peak of nitric oxide somewhat higher than the 40 ppm measured by the NOxBOX. The control animals should have received equal flow of nitrogen to completely mimic the iNO treatment.

5.2 Discussion of results

Specifics for each paper. The first two papers (**I, II**) shows that it is possible to combine iNO administration with nasal CPAP to term and premature infants. Newborns with moderate RDS treated with nCPAP before receiving exogenous surfactant acutely respond with a moderate improvement in oxygenation when exposed to 10 ppm iNO. The oxygenation response is proportionally greater in the more premature patients. There was no a change respiratory rate or arterial carbon dioxide tension indicating that respiratory drive was unaltered.

The environmental consequences for ICU room air from a single patient treated with iNO are negligible and well below the fluctuations in levels of oxides of nitrogen in ambient air (**III**). These results taken together make it feasible to plan future studies with neonates focusing on clinical outcome from early therapy with iNO. Meanwhile an increasing body of experimental evidence suggest that an earlier more prophylactic approach of iNO use might be of clinical value. As a matter of fact a large clinical study focused on the prevention of the development of BPD by prophylactic treatment of RDS with iNO to the very premature infants is about to start in Europe this year (B Jónsson, personal communication). This study includes both surfactant administration as well as subsequent administration of iNO (both MV and nCPAP) and we are eagerly awaiting the results of this very important clinical investigation.

Since patients with RDS are at risk of having too little and also easily inhibited endogenously produced surfactant, the possible impact of iNO at different concentrations and treatment times on surfactant function is an important safety consideration. iNO administration should always be studied as an adjunct therapy to administration of exogenous surfactant in RDS, and used as such it might have both additional and synergistic clinical effects and animal experimentation suggests that this is the case. Data from this thesis (**IV**) show

that a high dose iNO (100 ppm) for 4 hours causes a slight impairment of surfactant function in piglets. This effect is separate from the deleterious effects of NO₂. Prolonged exposure (24-48 h) to 40 ppm iNO in piglets receiving ventilatory support does not reproduce these findings. However the model itself tended to produce significant lung damage in the controls but not in the iNO exposed at the 24 h point as evidenced by worsening PaO₂ / FiO₂ ratio (**V**).

Future clinical aspects.

Before discussing the possible future implications of the combined use of nCPAP and iNO in RDS the author clearly wants to state that iNO-nCPAP is not an alternative to surfactant replacement therapy in RDS. Surfactant deficiency is beyond doubt the most central component of RDS and, thus, surfactant replacement should logically remain as the primary treatment. However as will be discussed below, the early combined use of InSurE nCPAP and iNO may have some added benefits in the treatment of premature infants with RDS both in the acute and the long-term perspective.

There are conflicting views on the clinical utility of the concept of InSurE, but in fact the use of both CPAP and surfactant are increasing in most countries, as discussed in more detail in section 1.1. However morbidity in survivors has however not decreased to the same extent. In the acute situation the addition of iNO to the InSurE-nCPAP concept could have a beneficial effect on coexisting pulmonary hypertension. It is well known that RDS infants that do not respond or only have a partial response to surfactant replacement also often suffer from a significant degree of pulmonary hypertension (38). In addition, premature infants have a lower nasal endogenous NO production than full term infants (105). Thus, adjunct use of iNO in such a situation could potentially reduce pulmonary artery pressure (PAP) and improve the response rate to surfactant administration by reducing both intra- and extrapulmonary shunting and also by off-loading the right ventricle and thereby improving overall cardiac output. If this would be the case the need for subsequent endotracheal intubation and MV due to treatment failure could potentially be further reduced. Since endotracheal intubation and MV are detrimental to the lung (22), any reduction in the need for this relatively drastic procedure and

therapy should be viewed as a considerable clinical advantage in the authors' opinion. Also the possibility to shorten the use of MV would be beneficial. Some patients previously treated with iNO and MV who no longer require ventilatory support may display rebound reactions at discontinuation of iNO. For these patients nCPAP with nitric oxide should be considered as an alternative to administering iNO by prolonging MV.

Any reduction in the occurrence of CLD/BPD in premature infants with RDS would thus be of great benefit not only to the patient and their families but would also result in substantial socio-economic savings.

No clinical data is to our knowledge currently available to corroborate the above hypothesis of a beneficial effect of adding iNO to surfactant replacement in RDS, supportive evidence for the use of such a combination has been shown in animal studies using various models of respiratory failure (e.g. surfactant depletion by repeated lung lavage, oleic acid injury, endotoxin injection, meconium aspiration) (12; 43; 140; 143). In an early investigation by our group, using an oleic acid injury model of RDS in rabbits, a significant improvement in oxygenation and survival was observed in animals treated with a combination of surfactant and iNO. The improvement in oxygenation and survival was not only evident when compared with the control group but also when compared with the use of NO or surfactant alone (143).

A subsequent study, using a surfactant depleted rabbit model, also showed that surfactant administration appears to be a prerequisite for an optimal effect of iNO (141). The pathophysiology of developing of BPD is still not fully understood. Inflammation (47) and also disturbed vascularisation of the lung (116) have however been proposed as major components of the disease process. Patients with BPD also develop pulmonary hypertension that has been shown to respond to iNO (58; 76). iNO has been found to beneficially influence inflammation (Flowchart, antioxidant effect of NO) (13; 102), angiogenesis, (119), lessen smooth muscle hypertrophy (70; 101; 107) and being a downstream mediator of VEGF to promote normal lung growth (116).

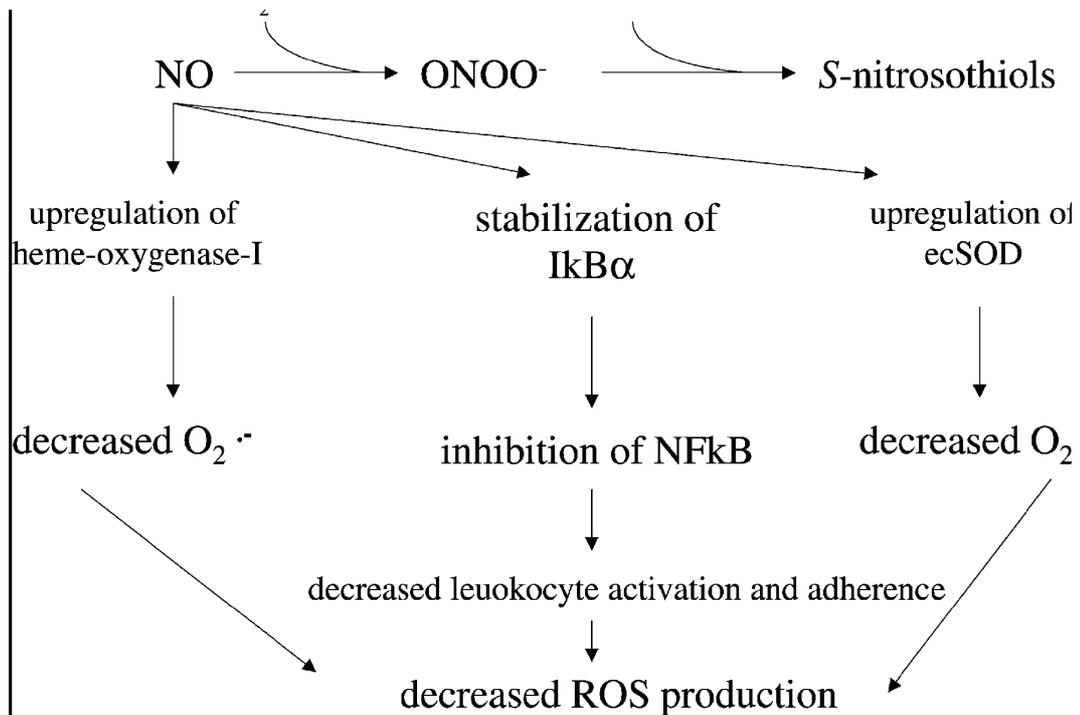
Recently a Texas research group demonstrated that pulmonary NOS expression is attenuated in a foetal baboon model of BPD (1) and suggests that the alteration in NOS expression may be an additional factor in the

development of BPD. Furthermore, the same group published a further baboon study in 2004 (of 14 days duration) showing that postnatal addition of iNO 5 ppm did have several beneficial effects that potentially can counteract the development of BPD (71). Apart from showing a reduction in PAP, greater lung compliance, improved expiratory resistance, a higher proportion of spontaneous DA closure and improved post mortem pressure-volume curves, it was further shown that lung DNA content and cell proliferation rate were increased in baboons treated with iNO. A most striking finding was also that lung growth over the 14 days duration of the study was preserved to equal that of what occurs *in utero* during the same time period.

Whether the above bears any clinical relevance can only be answered by future large-scale prospective, randomised clinical trials. However we have developed and validated an nCPAP system that can deliver iNO in an effective and from a working environmental aspect safe way, allowing for such large-scale multi-centre trials to be conducted.

Just as clinical use of exogenous surfactant has moved in the direction of prophylaxis and away from rescue, clinical use of iNO may face a similar development with an increased emphasis on lung development and anti-inflammatory action rather than just acute improvements in oxygenation and pulmonary vascular tone.

Antioxidant effect of NO



Nitric oxide capable booth of oxidant and antioxidant behaviour. The illustration exemplifies how NO has several pathways for antioxidant actions and protection of endothelial cells. These all lead to a reduction of the formation of radical oxygen species (ROS). Heme oxygenase –I is an indirect scavenger of $O_2^{\cdot-}$ by catalysing the formation of bilirubin which scavenges $O_2^{\cdot-}$. The activation of leukocytes is down regulated by inhibiting nuclear factor NFκβ formation through a stabilising effect on IκBα. NO induces the expression of extra cellular superoxide dismutase (ecSOD) that can to absorb $O_2^{\cdot-}$ thus limiting peroxynitrate production. When iNOS is activated NO production increases and more peroxynitrate can form.

From: Walford G. Nitric oxide in vascular biology (130)

6 CONCLUSIONS

The modified constant flow nCPAP system for concomitant delivery of nitric oxide has a low rate of NO₂ formation **(I)**. Stock gas cylinder concentration of 50 ppm NO has favourable properties allowing simplification of administration and monitoring in comparison with 1000 ppm as well as a reduced risk for excessive NO₂ formation during inadvertent over dosage of NO with lower stock gas concentrations that follow a more linear rate of NO₂ production.

Applying the system **(I)** to premature newborns with moderate RDS, results in significantly improved oxygenation especially in the more premature patients **(II)**. Respiratory rate and CO₂ levels remain unaffected in spontaneously breathing patients during a 30-min exposure. NO₂ levels predicted in the previous paper were accurate.

Nitric oxide inhaled through an open nasal CPAP system at 10 ppm contributes only to a small extent to background hospital room levels of NO_x **(III)**. As a point source, brief peaks of more concentrated but still low NO, are seen in the breathing zone of an attending staff person. The total release of a 20 litre cylinder with containing 1000 ppm NO at 150 bar, results in top levels of NO of 30 ppm and NO₂ of 0.8 ppm respectively. These levels are below the present short term exposure limits for NO and NO₂ in Sweden.

Exposure of spontaneously breathing sedated piglets to 100 ppm NO in air for 4 hours results in slightly impaired surfactant function with higher surface tension as compared to controls **(IV)**. This is not due to concomitantly formed NO₂. Piglets exposed to only 10 ppm NO₂ had a normal surfactant function.

Prolonged exposure to 40ppm iNO of sedated piglets results in no surfactant abnormalities and preserves features of lung tissue histology **(V)**. Compared to controls, iNO exposed animals fare better with lower lung injury scores and wet / dry ratios on model-related lung injury markers.

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Mariann Högman, under your expertise some 50 piglets have slept 8 hours during spontaneous breathing without a mishap, a world record?

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8 SVENSK SAMMANFATTNING

Andningstörning hos för tidigt födda barn (RDS): aspekter på behandling med inhalerad kvävemonoxid (NO), surfaktant och nasal CPAP

Inom nyföddhetsvården är andningsstörning (på engelska. *respiratory distress syndrome, RDS*), orsakad av brist på det ytspänningsnedsättande ämnet surfaktant i lungan, fortfarande ett stort problem hos för tidigt födda barn. Trots framgångar att ersätta surfaktantbristen med att ge surfaktant från djur (huvudsakligen gris) måste de för tidigt födda barnen ofta respiratorbehandlas. Behandlingen räddar liv men kan medföra ytterliggare lungskada. Tillståndet benämns Bronkopulmonell dysplasi (BPD), som definieras som kvarstående behov av syrgas över de första 30 dagarna och i vissa fall längre tid. Vissa barn med BPD kommer under de första levnadsåren att ha ökat behov av sjukhusvård på grund av luftvägsinfektioner och astmaliknande symtom. Detta växer oftast bort, men det finns även andra handikapp som möjligen är förknippade med syrgasbrist hos 30-70 % av dessa barn. I denna grupp är behovet av stödinsatser under skoltiden stort.

Hos svårt lungsjuka barn har inandad kvävemonoxid (iNO) används för att förbättra syresättning samt minska kärlsammandragning i lungan utlöst av syrebrist. Denna behandling startats sent i sjukdomsförloppet och ges endast till de svårast sjuka. Experimentell forskning på djur visar dock att tidigt insatt NO behandling är både antiinflammatoriskt samt leder till en mer normal lungutveckling. I lungan kan då iNO ersätta det kroppsegna NO som visats vara lågt vid för tidig födsel. Vår hypotes är att iNO givet under kontinuerligt positivt övertryck via en mask över näsan (nCPAP) skulle kunna minska behovet av intubation och respiratorbehandling hos RDS barn och därmed också kunna minska uppkomsten av BPD.

Målet med studierna var primärt att utveckla en administreringsmetod för att kunna kombinera iNO med nCPAP och göra behandlingen effektivare. Därigenom skulle intubation och respiratorbehandling kunna undvikas i större grad.

Ett nCPAP-system modifierades för NO behandling, för att få en låg bildning av giftig kvävedioxid, (NO_2) Vi mätte också NO_2 nivåer då olika koncentrationer av NO stamgas användes. I ett pilotförsök undersöktes de effekter som iNO-nCPAP har på syresättning, andningsfrekvens och arteriell koldioxidnivå hos 15 prematura barn med RDS som gavs 10 miljondelar (ppm) NO i inandningsluften under 30 minuter.

Risken för oönskad exponering med nitrösa gaser av personal under pågående NO-CPAP behandling studerades, eftersom CPAP kräver högre gasflöden än respirator och är ett öppet andningssystem. I en simulering av CPAP behandling med 10 ppm NO i ett intensivvårdsrum undersöktes halterna av NO och NO_2 runt kuvösen samt i rumsluften. Bakgrundsvariationen av nitrösa gaser i inluft mättes också under två veckor eftersom de kan variera kraftigt. I

en försöksmodell simulerades ett stort gasläckage för att mäta vilka NO / NO₂ koncentrationer en total tömning av en cylinder innehållande 10 liter 400 ppm eller 20 liter 1000 ppm NO ger i ett intensivvårdsrum.

Att ge behandling med den föreslagna kombinationen av iNO + nCPAP vid tillstånd som karakteriseras av surfaktantbrist motiverade att även undersöka NOs effekter på surfaktant. Vi undersökte friska spädgrisar som endera fick andas in 100 ppm iNO under 4 timmar (en mycket hög dos) eller 10 ppm NO₂ vilket tidigare visat sig ge lungskador (koncentrationen är ca 4 gånger högre än den NO₂ som bildas vid iNO 100 ppm). Slutligen studerades intuberande grisar som spontanandades med understöd av respirator och fick 40 ppm NO (dubbel maximal klinisk dos) under 24 eller 48 timmar. Både surfaktantfunktion och mikroskopiska förändringar i lungans utseende undersöktes.

Resultat

INO-nCPAP- systemet bildade endast låga koncentrationer av NO₂.

Användandet av 50 ppm cylindergas hade flera fördelar jämfört med 1000 ppm eftersom styr- och mätsystemen kunde förenklas och NO₂ bildningen begränsas ytterligare.

När barn fick inandas 10 ppm via detta system under 30 minuter ökade deras syresättning måttligt utan påverkan på andningsfrekvens eller blodets kolsyrenivå.

Tillskottet av NO och NO₂ i ett intensivvårdsrum där NO-CPAP behandling simulerades var mycket lågt jämfört med normalvariationerna av NO i inluft. Måttligt ökade nivåer kunde kortvarigt uppmätas då kuvörens luckor öppnades. Då en gascylinder med en koncentration av 1000 ppm tömdes på några minuter i rummet uppstod en topp på ca 30 ppm NO och 0.8 ppm NO₂ vilket är lägre än de teoretiska nivåerna beroende på bl.a. ventilationen.

Lungfriska spädgrisar som inandades 100 ppm NO under fyra timmar fick en måttlig störning av surfaktantets förmåga att minska utspänning. 10 ppm NO₂ gav inte denna effekt, varför störningen sannolikt förorsakas av NO. Någon surfaktantskada hos grisar som fått 40 ppm under 25-48 timmar kunde inte påvisas. Respiratorbehandlingen medförde en långsam lungskada i sig. De djur som fick NO behandling uppvisade lägre lungvikt, och något mindre lungskada jämfört med kontrollgruppen (endast sövda och ventilerade). NO togs upp i grisarna så att halterna av methemoglobin och andra nedbrytningsprodukter (nitrit och nitrat) ökade, vilket var förväntat.

Gynnsamma effekter på lungmognad och inflammation som iakttagits i djurstudier, kan eventuellt erhållas genom mycket tidigt insatt kombinationsbehandling med iNO-CPAP till för tidigt födda barn. Dessa effekter kan i framtiden visa sig vara lika värdefulla som sänkning av lungkärlstryck och förbättrad syresättning av iNO anses vara idag. De här genomförda studierna bidrar till en grund för framtida större klinisk prövning av sådan kombinationsbehandling. Liksom användning av surfaktant breddats mot mer förebyggande behandling, kan användning av iNO i kombination med CPAP möjliggöra liknande utveckling.

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