Cover Page for Protocol

Study Title:	Inhaled Nitric Oxide and Neuroprotection in Premature Infants
NCT Number:	NCT00515281
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SPECIFIC AIMS

With the advances in modern neonatal intensive care medicine in the last 20 years, survival of extremely preterm infants weighing less than 1500g (<3 lbs, 5 oz) has risen markedly. However, with this increased survival has come a marked increase in the number of infants with serious neurodevelopmental disabilities: Premature infants with birth weights less than 1500g who survive to go home are at significant risk for serious neurodevelopmental problems: cognitive and motor delays, blindness, deafness, and cerebral palsy. In a recent randomized, placebo-controlled clinical trial, we assessed whether giving mechanically ventilated preterm infants inhaled nitric oxide gas (iNO) for 1 week after birth decreased the incidence of death and chronic lung disease. An unanticipated outcome of that study[17] and a subsequent study of those infants at 2 year of age[14] was that premature infants treated with inhaled nitric oxide (iNO) have improved neurodevelopmental outcomes and physical growth at 2 years corrected age, compared with placebo-treated infants[14]. *INO therapy, therefore, appears to be a new treatment to protect the premature brain during development outside the womb*. **The overall goal of this application is understand the efficacy of iNO treatment in improving neurodevelopmental outcomes in at-risk premature infants.**

The improvement in neurodevelopmental outcome we reported was primarily due to a decrease in the risk of *cognitive* impairment[14], measured with the Bayley scales of Infant Development[2], a standardized tool for assessing neurodevelopmental outcome in this age group. As mentioned above, the initial hypothesis of the study was that iNO therapy would decrease the incidence of death and chronic lung disease. However, we found that the beneficial effect of iNO therapy on neurodevelopment was independent of important clinical factors known to affect neurodevelopmental outcome, including whether infants had chronic lung disease. Furthermore, the magnitude of improvement in neurodevelopmental outcome persisted after statistical adjustment for the presence and absence of intraventricular brain hemorrhage or periventricular leukomalacia (PVL, a softening of the white matter of the brain, due to reduced blood flow). Therefore, iNO therapy appears to be directly neuroprotective in ventilated, preterm infants. The number of children in each group having cerebral palsy, motor delay, blindness, or deafness did not differ between iNO- and placebo-treated infants.

Advances in neonatal intensive care medicine notwithstanding, we have not successfully developed neuroprotective therapies for these at risk preterm infants; iNO therapy, therefore, constitutes the first

intervention to allow the preterm brain to develop normally outside the womb. In our previous study, we treated babies for one week regardless of their level of prematurity. However, we do not know what duration of treatment will result in maximum neuroprotection, and will give the best neurodevelopmental outcome to the most infants. It is likely that maximal benefit of iNO therapy will occur with longer treatment, throughout the preterm period. *Our goal, therefore, is to determine whether iNO therapy given during throughout the preterm period results in further improved neurodevelopmental outcomes, and to understand the biological relationships between iNO treatment and neurodevelopmental outcome.* To achieve this goal, we propose performing a randomized, placebo-controlled, double-blind study of the effect of iNO therapy with gestational age-determined treatment length on neurodevelopmental and growth outcomes of preterm infants. Our Specific Aims are:

Specific Aim #1. Determine the effectiveness of iNO therapy on improving neurodevelopmental outcome in all O_2-dependent premature infants having birth weights < 1500g. *We hypothesize that iNO therapy will improve neurodevelopmental outcome and growth at 18 months postconceptional age compared with placebo treated infants.* In a double-blind, placebo-controlled clinical trial, premature infants being cared for in the Intensive Care Nursery at the University of Chicago Medical Center requiring any level of respiratory support will be randomized to receive iNO or placebo gas. Treatment will continue until infants *either* no longer require supplemental O_2 therapy *or* reach 33 weeks gestational age, whichever comes first. At 18 months postconceptional age, infants will have neurodevelopment assessed and secondary clinical outcomes measured. The relative risks of abnormal neurodevelopmental outcome in the two groups will be compared. The roles played by variables known to independently affect neurodevelopmental outcome, as well as variables known to mediate abnormal neurodevelopmental outcomes will be statistically assessed.

Specific Aim #2. Assess the mechanisms by which iNO therapy alters neurodevelopmental outcome in preterm infants. *We hypothesize that the amount of nitric oxide delivery to tissues will be positively correlated with neurodevelopmental outcome.* Using routinely collected arterial and venous blood from infants in this study, measurements of nitrosylated hemoglobin and nitrite will be made. Arterial-venous differences will be calculated and mean differences compared across treatment groups. Arterial venous

differences will be regressed against neurodevelopmental outcome. Plasma levels of cyclic guanosine monophosphate (cGMP), the intracellular second messenger induced by nitric oxide will also be measured and levels regressed against neurodevelopmental outcome.

BACKGROUND

1. Neurodevelopmental outcomes of premature infants

Improved survival of premature infants has generated intense interest in the quality of life of the survivors[6, 10, 20-23]. Premature infants have under-developed lungs, brains, digestive tracts, and immune systems. Neurological outcomes for these infants include brain injury (intraventricular hemorrhage, periventricular leukomalacia), blindness, deafness, chronic lung disease, developmental and motor delays and cerebral palsy. The incidence of each of these adverse outcomes increases as infants are born more prematurely. Complicating factors, such as respiratory distress syndrome also has a negative impact on these outcomes. Lower socioeconomic status (SES) also has a marked negative impact, with the risk of abnormal neurodevelopmental outcomes being significantly increased in families with lower SES[13].

2. Prior interventions to improve neurodevelopmental outcome

The need for effective interventions to improve neurodevelopmental outcomes for these infants has been a topic of concern for a long time. Randomized trials of indomethacin for closure of the ductus arteriosus was found to decrease the most severe form of bleeding in the brain (intraventricular hemorrhage, Grade IV)[11, 12], but a subsequent large randomized clinical trial was able to detect no differences in survival without neurosensory disability at 18 months corrected age[16]. Similarly, a number of trials assessed whether maternal Phenobarbital administration could decrease brain bleeding in very preterm infants < 1000g. While initial reports seemed positive, more modern trials have failed to show differences, either in the rate of severe brain bleeding or in the incidence of adverse neurodevelopmental outcome[3].

3. How does iNO therapy affect neurodevelopmental outcome in preterm infants?

In our previous single center, double-blind, randomized, placebo controlled study of 207 infants, we

demonstrated that iNO decreases the rate abnormal neurodevelopmental outcome in infants with birth weights <2000g receiving ventilator therapy for RDS. Our population group was predominantly African-American of low socioeconomic status. The original intent of this study was to assess role of iNO in decreasing death and chronic lung disease (a complication of prematurity and the need for mechanical ventilation). Nitric oxide was given at the dosage rate of 10 parts per million (ppm) for 12-24 hrs, then 5 ppm for up to 7 days, or until extubation. Infants were also randomized to receive either conventional ventilation or high frequency oscillatory ventilation (HFOV), a popular ventilatory approach believed by some to decrease chronic lung disease. We found no differences in the incidence of chronic lung disease and death between the groups receiving one of the two types of ventilator therapy. Our study concluded that nitric oxide in premature infants with respiratory distress syndrome decreased the incidence of chronic lung disease and death, consistent with our primary hypothesis. We also found that infants treated with iNO also had a markedly decreased incidence of the primary neurological complications of prematurity – serious bleeding in the brain (intraventricular hemorrhage) and strokes in the white matter of the brain (periventricular leukomalacia or PVL).

We then followed these infants after discharge for two years. We were able to maintain contact with 82% of those in the original study, a very high follow up rate for this population. In the follow-up study we found that iNO treated infants had decreased abnormal neurodevelopmental outcomes compared with placebo-treated infants. That beneficial effect persisted after statistical adjustment of the outcome for the presence of independent factors known to affect neurodevelopmental outcome: birth weight, gender, exposure to postnatal steroids, as well as factors that mediate abnormal neurodevelopmental outcome – intraventricular hemorrhage/PVL and incidence of chronic lung disease. We were able to conclude, therefore, that iNO has a beneficial, independent effect on neurodevelopmental outcome. We also found that iNO-treated infants grew better. They were only 0.5 standard deviations below the mean of full-term children, compared with more than 1 standard deviation for placebo-treated children.

There have been two other large placebo-controlled, randomized, double blind studies of iNO therapy in premature infants. In a multi-center trial, Kinsella and colleagues at the University of Colorado[8] randomized 793 infants at 34 weeks gestation or less requiring mechanical ventilation to receive 5 ppm of iNO for 3 weeks. They reported no difference in BPD in the overall cohort. However, infants weighing 1000-1250g who received

iNO had a significantly reduced incidence of chronic lung disease compared with the placebo-treated group. Most excitingly, for the entire cohort, the incidence of the combined end point of IVH, PVL and ventriculomegaly (all signs of brain injury) was significantly reduced in iNO treated group over the placebo-treated group. This study provides support for our single center study that iNO therapy reduces overall risk of brain injury. It is also of importance to note that iNO was given for periods up to three times as long as our study, and without any adverse outcome.

Ballard et al[1] from the Children's Hospital of Philadelphia reported on their randomized double blind placebo-controlled, multi-center study of 582 infants weighing < 1250g who were on mechanical ventilation or continuous positive airway pressure (CPAP). In that study iNO was started later, at between 7 and 21 days of life. In contrast to our study, iNO was begun at a higher dose (20 ppm), then weaned first at 96 hrs of treatment and then weekly. Patients were treated for a minimum of 24 days. The primary outcome was survival without chronic lung disease. iNO was shown to decrease the incidence of survival without BPD. However, neurodevelopmental outcomes were not evaluated. However, this study confirms our initial study's findings that iNO decreases BPD. Taken together these two studies provide confirming evidence that, in preterm infants, iNO therapy decreases BPD and brain injury.

4. What are the mechanisms by which iNO therapy alters neurodevelopmental outcome in preterm infants?

In studies of brain injury in animals, nitric oxide (NO) has been associated with neuroprotection, mostly likely through actions on the blood vessels of the brain. After inhalation, iNO crosses the alveoli (air sacs) of the lung and enters the circulation, where it is bound to hemoglobin. It had been believed that NO bound to hemoglobin was inactivated. How, then could inhaled NO be delivered to target tissues, such as the brain? New data indicate that hemoglobin may act as a delivery system for NO to target tissues[4, 5, 24]. Neuroprotection may be due to actions of NO on blood vessels in the brain, with its ability to increase blood flow to injured areas of the brain.

In the laboratory component of this study, we propose to measure NO metabolites in the blood of our study babies treated with iNO or placebo. Recent data now suggest that NO, both administered as well as naturally

produced, is transported in blood to target tissues, where it can increase local blood flow. This has been documented in humans, where inhalation of NO was found to increase blood flow in the arm of exercising volunteers[5]. Furthermore, NO has been shown to be carried on the hemoglobin molecule in red blood cells – the hemoglobin becomes nitrosylated, carrying an NO molecule[4]. Thus, iNO markedly elevates levels of total nitrosylated hemoglobin in human volunteers[24]. Most importantly, there is a marked difference in the amount of nitrosylated hemoglobin between the arterial circulation carried to the tissues, and the venous circulation carried away from the tissues[4]. This difference indicates that inhaled NO delivered in the arterial blood is being removed from hemoglobin in the tissues, consistent with its effect in organ capillary beds. In addition to nitrosylating hemoglobin, plasma nitrite also appears to be a source of bio-available NO. Nitrite, generated from the reaction of NO with oxygen, can be converted back to NO by means of naturally occurring enzymes in the body. In fact, similar to levels of nitrosylated hemoglobin, large differences in arterial and venous nitrite levels have been documented in humans, and has been shown to be bioactive[5].

In the laboratory component of this study, we propose to measure NO metabolites in blood in children treated with iNO or placebo, and calculate the NO delivery to tissues using arterial and venous measurements. We will describe the time course of these metabolites during the course of iNO therapy, and correlate the level of NO metabolites with neurodevelopmental outcome.

SIGNIFICANCE

Despite much research into potential neuroprotective treatments for premature infants, the risk of abnormal neurodevelopmental outcome persists. Concurrently, the number of surviving preterm infants is rising because of advances in neonatal intensive care medicine. Inhaled nitric oxide is the only treatment shown to improve neurodevelopmental outcome in preterm infants. The results from the proposed study will establish whether iNO is effective in improving neurodevelopmental outcome for the vast majority of premature infants, i.e. those infants needing some respiratory support. If our hypothesis is upheld, iNO it has the potential to markedly impact the quality of life for these babies and their families.

RESEARCH PLAN

Overall Experimental Plan: The study is a single-site, randomized, double-blind, placebo-controlled trial of inhaled nitric oxide therapy in all preterm infants with any respiratory disease who weigh <1500g at birth. The trial is designed to treat these infants with nitric oxide from birth to 33 weeks gestation – a critical period of brain development in preterm infants. Our primary objective is to compare neurodevelopmental outcomes between our iNO and placebo-treated patients. We also wish to study the mechanisms underlying the protective effect of iNO, by assessing the relationship between nitric oxide delivery to tissues and neurodevelopmental outcome.

The major differences between this study and our previous study are:

1) Inclusion of all preterm infants requiring at least supplemental oxygen therapy. Approximately half of all infants weighing <1500 at birth do not require mechanical ventilation with a breathing machine, which was one criterion for inclusion in our previous study. However, these infants are still at risks for abnormal neurodevelopmental outcomes. By including all infants requiring at least supplemental oxygen by nasal cannula, we hope to obtain information regarding the potential beneficial effects of iNO applicable to the majority of at-risk preterm infants.

2) Gestational age-dependent duration of iNO therapy. In our previous study, infants were treated with iNO or placebo for 7 days (if still on mechanical ventilation). Consequently, infants received iNO therapy during different periods of brain development: for example, infants of 24 weeks gestation (16 weeks early) received iNO during a period during brain cells were being rapidly produced, while infants of 30 week's gestation received iNO after brain cells had been made and were now forming connections. In this study, our goal is to provide iNO during the entire time the premature brain is developing outside the womb. Therefore, infants will be treated from birth until 33 weeks gestation, so that less mature infants will be treated for a longer time.

3) Specific design of the study to assess neurodevelopmental outcome. Our previous study had been designed to assess the effect of iNO treatment on the incidence of chronic lung disease and death; we assessed these infants' neurodevelopmental outcomes because of our unanticipated finding of decreased brain bleeding and softening. This study has as its primary outcome the incidence of abnormal neurodevelopmental outcome.

4) Laboratory-based Specific Aim to assess the mechanisms of the iNO-induced neuroprotection.

Specific Aim #1. Assess the role of iNO therapy on neurodevelopmental outcome in all oxygen-dependent premature infants having birth weights < 1500g.

(i) Rationale. We have previously found that brief (1 week) treatment of ventilated preterm infants reduces abnormal neurodevelopmental outcome. Because brain development continues throughout gestation, we hypothesize that iNO therapy will be protective to the preterm brain if given from birth until the risk for abnormal neurodevelopmental outcome is similar to that of term infants.

(ii) Study design. We have designed a randomized, placebo controlled, double blind study of iNO therapy during Intensive Care Nursery stay, followed by blinded neurodevelopmental assessment at 18 months corrected age, a commonly used developmental endpoint.

(iii) Patients. All premature infants cared for in the Intensive Care Nursery at the University of Chicago Medical Center, who are less than 72 hours old, weighing less than 1500 grams at birth, and requiring any respiratory support, will be eligible for the study. Enrolled infants are anticipated to have the same demographics as we have previously reported[14]: 52% male, 70% African-American, 45% single parent household. Potential subjects will be identified and informed consent sought, if possible, prior to birth, otherwise, within the first 3 days after birth.

(iv) Exclusion criteria. Those infants with severe congenital anomalies, including major congenital heart disease, or major kidney, lung or brain malformations will be excluded, as will be those infants with a genetic syndrome. Infants judged by the physician to not be viable will be excluded from the study, as will extremely sick infants requiring very high ventilatory pressures (Oxygen Index ≥ 20). This latter group is excluded because of a previous study by Van Meurs[19] which showed that infants in this category are made worse by inhaled nitric oxide.

(v) Patient randomization. Randomization will be performed as we have previously published[17]. To keep birth-weight distribution comparable in the two groups, we will use five 250-gram-birth weight strata for randomization. To ensure equal enrollment in the two groups, infants will be randomly assigned within each stratum, according to a permuted block design to receive inhaled nitric oxide (INOmax®, INO Therapeutics) or

oxygen placebo. This strategy provides a balanced group assignment with every 4 enrollments.

(vi) Patient enrollment at study entry procedures. We will enroll patients and enter them into the study as we have previously published[17]. Briefly, potential subjects will be identified, prenatally if possible, and the parents approached for informed consent. With consent obtained, and the infant born, admitted, and verified to meet inclusion criteria, a sealed envelope containing the randomized treatment assignment, appropriate identifying labels, etc., will be pulled and opened by the respiratory therapist. Documentation of the treatment assignment and patient data will be recorded by the respiratory therapist in the study book (kept in the respiratory therapy room), and an INOvent® brought to the bedside. The INOvent will be set to deliver either iNO or oxygen according to the treatment randomization and then covered to conceal the treatment group from the caregivers and investigators.

(vii) Treatment. iNO or placebo (oxygen) will be given 5 ppm while ventilated or on continuous positive airway pressure (CPAP) and 10 ppm while on nasal cannula oxygen. In infants receiving iNO by nasal cannula, iNO concentrations are set to 10 ppm, because previous studies have shown that delivery 10 ppm by nasal cannula results in nasopharyngeal concentrations of about 5 ppm[9]. These dosages will continue until the infant no longer needs supplemental oxygen or reaches 33 weeks post conceptional age. All infants will have iNO delivery devices (INOvents) at the bedside to deliver either iNO or O_2, and will be shrouded to hide from caregivers the nature of the gas being delivered. Only respiratory therapy and data safety monitor to know of gas assignments.

(viii) Data Collection. Clinical data (fluids, medications, diagnoses, ventilator settings) will be collected daily by our research professional.

(ix) Sample size. In our previous study of infants with RDS[17], iNO treatment cut the risk of abnormal neurodevelopmental outcome by half. The baseline incidence of abnormal neurodevelopmental outcome is 40% in this age group by published NICHD data[7]. Therefore to see a decrease of abnormal neurodevelopmental outcome to 20% (half of baseline incidence) with 80% power, 82 babies are needed in each arm of the study, for a total of 164 babies. We have previously published an overall mortality rate for intubated infants < 2000g in our nursery of 23%[17]. Because we are including non-intubated infants, we

estimate a mortality rate of about 18%. Therefore, to ensure sufficient infants surviving to follow up, we anticipate needing to enroll approximately 215 babies over 18 months. With a historic 80% follow-up rate, we will need a total of 268 infants. Every year we admit approximately 175-200 infants with birth weights < 1500g. We therefore anticipate enrollment taking as long as 18 months. Our non-consent rate historically is < 10%.

(x) Standards of neonatal intensive care. Because we have shown no effect of ventilatory strategy on neurodevelopmental outcome, there will be no external control of ventilatory modality. Because this is a single-site study, variability in practice exists only between our eight member group of neonatologists, with constant care provided by nurse practitioners, residents, and nursing staff.

(xi) Safety monitoring. As per our clinical routine, methemoglobin is regularly monitored with arterial blood gases. Although we and others have shown that iNO therapy is safely used in preterm infants, interim safety analyses will be performed when 50% of patients enrolled. Deaths will be compared, as will the incidence of severe intraventricular hemorrhage, and tests of significance made with Chi-square. The study will be stopped if the incidence of either index in the iNO groups is significantly higher than in placebo groups.

(xii) Primary Outcome. As we have published[14], we will measure abnormal neurodevelopmental outcome at 18 months corrected age. Three disability levels will be assessed: disability (cerebral palsy, blind in both eyes, or deaf in both ears); delay (score on Mental or Psychomotor Developmental Index of the Bayley Scales of Infant Development < 70 without a disability) or normal (without disability or delay).

(xiii) Secondary Outcomes. Other neurodevelopmental outcomes to be measured include the incidences of disability or delay; mean cognitive (MDI) and psychomotor (PDI) function scores will be obtained and infant growth charted. Important clinical diagnoses known to affect the risk of adverse neurodevelopmental outcomes will be tracked: respiratory distress syndrome, bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular hemorrhage (with grade), periventricular leukomalacia, retinopathy of prematurity, and failed hearing screen. Finally, detailed clinical data will be tracked on a daily basis, including fluids, ventilatory status, blood gases, feedings, etc.

(xiv) Measures to ensure long-term neurodevelopmental follow-up. The very high follow-up rates we have previously published require intensive efforts of a number of teams following a patient's discharge, including social services and the research staff. All preterm infants will be followed in our high risk follow-up clinic. This clinic is unusual, in that it provides routine, well-child care to its babies, as well as subspecialty care and neurodevelopmental care. Providing routine care provides an important incentive to parents to come to clinic, since they only have one appointment to make. In addition, we provide regular reminders of the role of the University of Chicago Medical Center in the lives of our families: patients receive holiday and birthday cards, and personalized invitations to our regular patient reunions. Finally, families who come for follow-up visits are given gifts and their travel expenses are reimbursed. Families are rewarded at a 9 month follow-up visit and at the 18 month follow-up visit. With this approach, our drop-out rate is less than 15% -- very low for this population by published reports.

(xv) Assessment of neurodevelopmental outcome. As we have previously published[14], at 18 months post conceptional age, developmental screening with Bayley exams will be performed in our High Risk Follow-up Clinic by Dr. Michael Msall, who will also perform neurological examinations to identify those infants with cerebral palsy. Other assessments will include measurements of physical growth and review of routine visual and hearing examinations to determine blindness or deafness. Questionnaires assessing socioeconomic status and self report of racial ethnicity will be administered. All examiners will be unaware of treatment regimen assignment. In our previous studies, our follow-up rate of >80% is very high by published standards, especially for an inner city community such as we serve. We anticipate that rate will continue for the current study.

(xvi) Data analysis. As we have previously published[17], data will be analyzed as "Intent to Treat.". Clinical and demographic variables between groups will be compared with Fisher's exact test for categorical variables and Wilcoxon rank-sum tests for continuous variables. Primary outcomes will be analyzed with generalized linear models to obtain relative risks of primary outcome in the two groups. Effects of potential confounders will be adjusted for in the model: birth weight, sex, socioeconomic status, as well as for potential the intermediate variables: prolonged exposure to postnatal steroids, presence of chronic lung disease, severe IVH, or periventricular leukomalacia (PVL). Further analyses of Bayley scores, both cognitive (MDI) and psychomotor (PDI) will be performed.

(xvii) Anticipated results and critique. We anticipate that infants treated with iNO will demonstrate significantly decreased incidences of abnormal neurodevelopmental outcomes and will also demonstrate significantly increased cognitive abilities, as demonstrated by MDI scores. We further anticipate that these effects will persist after statistical adjustment for factors independently predisposing to abnormal neurodevelopmental outcome, such as male gender and gestational age, as well as adjustment for factors potentially mediating adverse neurodevelopmental outcome, such as chronic lung disease, intraventricular hemorrhage, or PVL.

In our previous study, we found that some infants tolerated our standard weaning protocol from iNO poorly, requiring much slower weaning and therefore a longer time on very low concentration (1-2 ppm) of NO. One mechanism underlying this minor complication is decreases in native NO production by blood vessels of the lung during prolonged periods of iNO delivery. Therefore, it is conceivable that extremely preterm infants, who will, by design, receive iNO for up 8 or 9 weeks, exhibit intolerance to weaning off iNO. Accordingly, we will wean patients by 1 ppm every 12-24 hrs, the protocol which served us well in the previous study in this setting.

Specific Aim #2. Assess the mechanisms by which iNO therapy alters neurodevelopmental outcome in preterm infants.

(i) Rationale. We have previously found that iNO therapy reduces the risk of abnormal neurodevelopmental outcomes independently of the level of prematurity or complications of prematurity known to affect neurodevelopmental outcome. Therefore, it appears that iNO has direct, beneficial effects on the preterm brain and its response to the stress of existence outside the womb. Previously, iNO was believed to be inactivated in the blood vessels in the lung, by binding to hemoglobin in the blood. However, there is increasing evidence that hemoglobin binding does not inactivate NO permanently, but rather transports it for delivery to target tissues[4, 5, 24]. The goal of this Specific Aim, therefore, is to assess the amount of delivered NO that is transported to our patients' tissues, measure the amount of NO activity in our patients' blood, and examine the relationships between these measures, the iNO concentrations administered, and the patients' neurodevelopmental outcomes.

(ii) Study Design. Measurements of nitrosylated hemoglobin from arterial and venous blood samples will be made on each patient at defined points during the hospitalization: in the first 48 hrs following initiation of study gas (iNO or placebo), after 1 week of treatment, after 2 weeks of treatment. In infants who are treated longer than 2 weeks (anticipated to be all infants born before 31 weeks gestation, since they will likely still require some respiratory support), weekly measurements of venous nitrosylated hemoglobin will be made. Cyclic GMP will be measured once, after 1 week of therapy. For each patient, arterial and venous values will be compared, to identify the amount of NO that is delivered to tissues, and these values plotted over time. Mean delivered amounts will be compared across treatment groups, and individual values correlated with neurodevelopmental outcome. Cyclic GMP levels, a measure of nitric oxide activity, will be averaged within groups and treatment groups compared.

(iii) Source of blood. This protocol does not require for blood draws outside the regular scope of practice. Samples will be taken from regularly scheduled arterial and venous draws, and leftovers from these samples used.

(iv) Measurement of nitrosylated hemoglobin. The S-nitrosylated hemoglobin will be measured using chemiluminescence, a technique we have used previously in the measurement of inhaled NO in tracheal air[15]. Analysis will be performed according to published methods[5]. Briefly, leftover blood from routine blood draws (arterial and venous) will be placed into EDTA collection tubes and taken to the lab, and centrifuged at 750xg for 5 min. Aliquots of plasma will be taken and stored at -80º for assays of nitrite and cGMP (see below). The red blood cell pellet will be removed, washed, flash-frozen on dry ice and stored at -80º C in a laboratory freezer. At the time of assay, the pellet will be rapidly thawed and pretreated with a 100-fold excess of KCN and $K_3Fe(CN)_6$, to remove No from heme while preserving the S-nitrosothiol bond. After pretreatment (30 min), 500 µl will be passed through a Sephadex G25 column to remove contaminating nitrite and small nitrosothiols. Samples will then be reacted with I^-_3 to release NO from S-nitroso-hemoglobin, and assayed by chemiluminescence. Results will be calibrated against S-NO-glutathione standards.

(v) Measurement of plasma nitrite. Plasma nitrite will be measured using a commercially available kit (World Precision Instruments, Sarasota, FL) and a spectrophotometer, available in the laboratory. A colorimetric reaction is generated, and the absorbance is read at 540 nm.

(vi) Measurement of cGMP. Guanosine 3'-5' cyclic monophosphate will be measured in plasma using a commercially available competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). The detection limit of this assay is about 6 pmol/ml, but acylation of the standards and samples will increase the sensitivity by 10-fold. Plasma proteins will be precipitated with ethanol and removed by centrifugation. The supernatant will be dried under nitrogen and re-suspended in diluted sample buffer. The assay is performed in 96-well format and after 18 hrs incubation at 4° C; Ellman's reagent is reconstituted and added to the wells. Absorbance is read 90 min later at 405 nm. Results will be calibrated against standards assayed simultaneously.

(vii) Data Analysis. Arterial-venous differences in S-nitroso-hemoglobin and plasma nitrite levels will be calculated on a patient-by-patient basis. Mean levels within groups will be compared across groups, and statistical significance tested with ANOVA[18]. Individual values of A-V differences will be compared with neurodevelopmental outcome on a patient-by-patient basis and assessed with regression techniques. Similar analyses will be performed with cGMP values.

EXPECTED IMPACT OF THE PROJECT.

Although there is great interest in the neonatal community about our prior finding of improved neurodevelopmental outcome and growth among premature infants treated with nitric oxide (see attached newspaper articles), additional corroborative data from a study specifically designed to examine the effect of nitric oxide therapy on neurodevelopmental outcomes is required to change current recommendations for neonatal care. If supportive, this specifically designed project would provide that corroborative data, such that we and other authorities could recommend that routine care of premature infants include nitric oxide treatment for improved neurodevelopmental outcomes. Consequently, this project would be expected to provide a marked improvement in neurodevelopmental outcome for the thousands of preterm babies born in the US every year.

PLANS FOR ACKNOWLEDGING FOUNDATION SUPPORT FOR THE PROJECT.

All papers reporting data from this study will specifically acknowledge the support of the Gerber Foundation. The text will say: “Funded by a grant from the Gerber Foundation.” Similarly, all talks, abstracts, and posters will contain specific acknowledgement.

Appendices

A. Project schedule/Timeline of events

Time	Project Stage	Activity
Year 1 month 1	Begin patient enrollment	randomize to NO or placebo
	Begin assessment of NO delivery and metabolism during hospitalization	measurement of arterial and venous nitrite and nitrosylated hemoglobin
year 1 month 3	anticipate 1st discharges to home - assess complications of prematurity potentially affecting neurodevelopmental outcome	Routine head ultrasounds Routine head MRI at discharge Classification of chronic lung disease
year 1 month 9	Interim safety analysis	Comparison of death rates and rates of Grade IV IVH between groups
Year 2 month 7	End patient enrollment	
Year 2 month 7	Begin assessment of 18 month neurodevelopmental outcome	Bayley scales Neurologic exam Assessment of CP status Assessment of Blindness Assessment of hearing
Year 3 month 12	End 18 month neurodevelopmental outcome assessment	

B. Collaborations/Linkages.

An ongoing collaboration will be made with the University of Chicago Neonatal Follow-up Clinic in which our infants will be seen for routine follow-up and neurodevelopmental assessment. Particularly important, will be our collaborations with Susan Plesha, our neurodevelopmental assessment specialist, who performs the Bayley developmental assessments on all our babies, as well as with our social work staff. Our social work staff's involvement with our subjects' families has been shown to be very helpful in keeping contact with them, and ensuring our very high follow-up rates in this economically depressed population.

C. Budget

D. Informed Consent Documentation

THE UNIVERSITY OF CHICAGO
The Division of the Biological Sciences. The University of Chicago Hospitals

CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH PROTOCOL

Protocol Number: _____ Patient Name: _____
Hospital Number: _____

Title of Protocol: NITRIC OXIDE AND NEUROPROTECTION (PREMATURE INFANTS)

Doctor(s) Directing Research: Michael D. Schreiber, M.D. Phone: 773-702-6210
Jeremy D. Marks, Ph.D. M.D.
Michael Msall, M.D.

You are being asked to have your baby participate in a research study. The doctors at The University of Chicago Hospitals and The Division of Biological Sciences study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

I. NATURE AND DURATION OF PROCEDURE (S):

Your premature baby has an illness, which results from the immaturity of the lung to exchange adequate amounts of oxygen into your baby's blood. Some medical investigators have found that the breathing a gas, called nitric oxide, causes more blood to travel through the lungs, and results in more oxygen in the blood. Nitric oxide, which occurs normally in the body, relaxes blood vessels. By using higher than normal nitric oxide concentrations, the blood vessels in the lung may relax further and improve oxygenation. We have used nitric oxide to successfully treat preterm newborn babies who need to be on a breathing machine, and have found that babies treated with nitric oxide not only have fewer complications of their lung disease, but may have improved neurodevelopmental outcomes. This study is specifically designed to determine whether nitric oxide improves neurodevelopmental outcomes in premature babies with some difficulty breathing, whether or not they need mechanical ventilation.

If your baby is assigned to receive inhaled nitric oxide, the amount of gas will be carefully controlled and adjusted to the amount that best improves the function of your baby's lungs. Your baby will remain on inhaled nitric oxide until s/he reaches 33 weeks of gestation or no longer needs supplemental oxygen.

After being discharged from the hospital, your baby will be seen in our High Risk Follow-up Clinic at 9 and 18 months of corrected age. He or she will be examined and will have developmental testing done by our developmental pediatrician. No invasive testing will be performed.

II. POTENTIAL RISKS AND BENEFITS: Based on the limited human and animal studies, in conditions similar to those encountered by your baby, no serious side effects of inhaled nitric oxide, in the concentrations delivered, have been demonstrated. Although not reported in humans, high concentrations of nitric oxide theoretically could cause bleeding problems. In addition, high concentrations of nitrogen dioxide, a by-product of nitric oxide, could cause lung injury. The precise concentration of nitrogen dioxide is continuously monitored and maintained within accepted guidelines. As is the case with any new drug, unknown side effects could occur. These potential risks are small compared to the risks of current conventional therapy. Determining which treatment will be most effective with the least side effects is the purpose of this study. The results will not to be known until after the study is completed and the data have been analyzed. Because your baby will receive pain medication and/or sedatives, no discomfort from this study should be experienced. Your baby's records will be kept as confidential as is possible within the law.

FINANCIAL CONSIDERATIONS Study gas and equipment will be provided free of charge. There will be no additional costs to you or your insurance carrier related to this study. Follow-up examinations will be performed at no additional cost to the family. Travel and meal allowances will be provided.

BENEFITS OF PARTICIPATION The potential benefits are that the treatment your baby receives may prove more effective than the currently available treatments and may prove to be helpful in preventing or ameliorating acute pulmonary injury which leads to the development of chronic lung disease.

III. POSSIBLE ALTERNATIVES: The alternative is to continue standard treatment using oxygen, mechanical ventilation, and other medications. Such currently used standard therapy is moderately successful in many cases.

AGREEMENT TO CONSENT

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available at The University of Chicago Hospitals. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. Records of study participants will be identified by code numbers. Results will be compiled as aggregate data. When required by law, the records of this research may be reviewed by applicable government agencies including the Federal Food and Drug Administration. The study sponsor may receive records of this research but will have no access to the names of the patients.

I understand that in the event of physical injury resulting from this research, The University of Chicago Hospitals will provide me with free emergency care, if such care is necessary. I also understand that if I wish, The University of Chicago Hospitals will provide non-emergency care, but the Medical Center assumes no responsibility to pay for such care or to provide me with financial compensation.

I, the undersigned, hereby consent to participate as a subject in the above described research project conducted at The University of Chicago Hospitals. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor(s) listed above. If I have questions concerning my rights in connection with the research, I can contact the Institutional Review Board, at 773-702-1472.

After reading the entire consent form, if you have no further questions above giving consent, please sign where indicated.

Doctor:_____ _____

Signature of Parent or Guardian

Witness:_____ Date: __/__/__ Time: _____

E. CV's of Principal Investigators

F. References

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