

Enteral L citrulline supplementation in preterm infants – safety, efficacy and dosing

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SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations and ICH guidelines.

Site Investigator: *

Signed: Snigdha Bhatia, M.D. Date: 06/02/2019
Name
Title

** The protocol should be signed by the local investigator who is responsible for the study implementation at his/her specific site; i.e., if Investigational New Drug study, the individual who signs the Form FDA 1572.*

1.0 Background

Bronchopulmonary dysplasia (BPD) is the most common chronic lung disease in preterm infants (PI). BPD is defined as requirement for additional oxygen at 36 weeks corrected gestational age (CGA) in infants born with a birth weight < 1500 g and GA < 32 weeks. Pulmonary hypertension (PH) develops in 25-37% of patients with BPD and is associated with significant morbidity/mortality(1). Multiple factors contribute to the development of BPD associated PH (BPD_PH). Preterm birth causes disruption in pulmonary vascular growth that leads to decreased vascular surface area that increases pulmonary vascular resistance (PVR). Increased PVR leads to altered vasoreactivity and structural remodeling with intimal hyperplasia and increased muscularization of the small pulmonary arteries. There is no definite treatment for BPD_PH.

Nitric Oxide: Nitric Oxide (NO) is a potent pulmonary vasodilator. Endothelial Nitric oxide synthase (eNOS) mediates production of NO from L-Arginine. L-citrulline is a precursor for L-arginine. L-Arginine is a precursor of nitric oxide (NO). Nitric oxide (NO) is produced in the pulmonary arterial endothelial cells (PAECs) from L-arginine by an enzyme endothelial nitric oxide synthase (eNOS). NO is a signaling molecule which leads to dilatation of lung blood vessels and decreases pulmonary vascular resistance, leading to decreased pulmonary hypertension. L-citrulline is an end-product of this reaction and it recycles back to form L-arginine via a two-step enzymatic pathway (figure 1) involving arginosuccinate synthase (ASS) and arginosuccinate lyase (ASL).

Arginine and citrulline in infants with BPD_PH: In infants with BPD_PH, there are decreased levels of L-arginine & L-citrulline with decreased production of NO (measured by urinary nitrates & nitrites) leading to increased pulmonary vascular resistance. It is suspected that genetic variations in enzymes involving citrulline synthesis may contribute to the susceptibility to develop pulmonary hypertension(2). A cross-sectional pilot study by Montgomery et al reported that L-citrulline levels < 29 $\mu\text{mol/L}$ predicted BPD_PH with 100% sensitivity and 75% specificity(3).

Supplementation of citrulline and PVR: Fike et al showed that oral supplementation with L-citrulline increases vascular NO production and attenuates the development of elevated pulmonary vascular resistance (PVR) in a newborn piglet model of chronic-hypoxia induced pulmonary hypertension(4). In a newborn rat model of hypoxia-induced BPD, subcutaneous injection of L-citrulline decreased pulmonary vascular remodeling and reduced right ventricular hypertrophy (both of these are markers of pulmonary hypertension)(5). A Phase 2 clinical trial of L-citrulline in pediatric patients with sickle cell disease showed decreased vascular complications and improvement in overall well-being(6). L-citrulline has been used in post cardio-pulmonary bypass patients and it has been shown that post-operative pulmonary hypertension did not develop in children who had naturally elevated citrulline levels or who achieved plasma levels greater than 37 $\mu\text{mol/L}$ with oral citrulline supplementation(7).

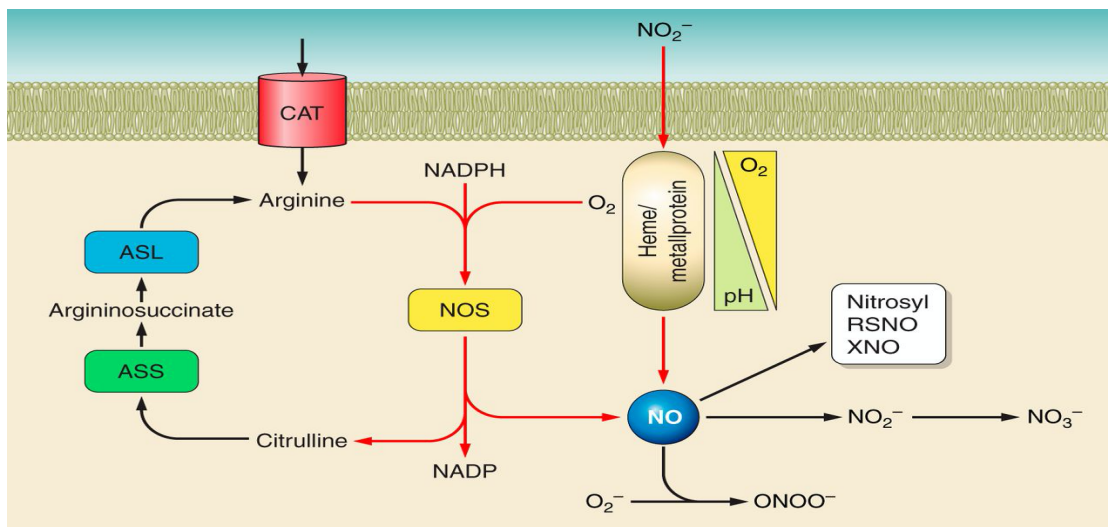


Figure 1. Urea cycle. Adapted from American Journal of Physiology, March 2015.

Why oral L-citrulline: In in-vitro studies, L-arginine promotes NO production (See Figure 1) but in in-vivo studies (animal models of PH), oral L-arginine did not increase NO production due to hydrolysis of L-arginine by *arginases* present in enterocytes in gastrointestinal tract and hepatocytes, leading to decreased bioavailability of oral L-arginine. Alternatively, oral L-citrulline (precursor for L-arginine figure 1) can be used to increase L-arginine & NO production. After being produced and released into the circulation by enterocytes, L-citrulline passes through the liver without major metabolism. Cells in the proximal tubules of the kidney take up and metabolize some of the circulating L-citrulline into L-arginine in urea cycle via enzymes argininosuccinate synthase and argininosuccinate lyase. Therefore, a significant portion of the L-citrulline produced by enterocytes reaches the systemic circulation as compared to L-arginine. PAECs express the enzymes ASS and ASL and can use L-citrulline to produce NO. There is some evidence suggesting that co-localization of these enzymes with eNOS in the plasmalemmal caveolae (invaginations of plasma membrane in the pulmonary arterial endothelial cells) may allow L-citrulline-induced increases in L-arginine to be directly channeled to eNOS rather than equilibrating with intracellular levels(2).

In-vitro and In-vivo studies of L-citrulline supplementation in newborn piglets showed increased NO production and decreased PH in newborn piglets. These studies also showed that treatment with L-citrulline reduced superoxide generation, increased NO production as well as helped with eNOS re-coupling which is the active form of eNOS (dimeric form of eNOS is the active form).

Arginine as essential amino acid: Under routine conditions arginine is not an essential amino acid but is considered an essential amino acid in neonates, particularly under stress, infection and prematurity. In neonatal period (suckling period), the major source for endogenous arginine biosynthesis is from citrulline in the intestine.

Source of L-arginine in preterm infants: Routinely, extremely premature infants receive nutrition as total parental nutrition (TPN i.e. infants get infusion of protein, fat and carbohydrate via central venous line) that contains L-arginine (approximately 1mg/1mL) to metabolize ammonia via urea cycle. PIs receive adequate amount of intra venous arginine from TPN. Routinely, PIs are started with small volumes of enteral feeds which are increased slowly overtime. TPN is slowly decreased as enteral feeds are increasing. As the TPN is going down, intra venous L-arginine intake also drops down and ultimately when the PI are off TPN, they don't get any IV supplemental L-arginine.

Why long term IV arginine not possible in preterm infants: In PI, the major cause of late onset infection (after 7 days of age) is central line related infection that is why central line is taken out as soon as infant reaches full feed.

Why oral citrulline: Enteral feeds (formula as well as breast milk) is poor source of arginine. Once PIs are on full enteral feed, an enteral feed is the only source of arginine. Interestingly, 40% of enteral arginine gets metabolized by arginase enzyme present in intestine. **We speculate that plasma levels of arginine drop once TPN is discontinued and infants are on full feeds.** Oral L-arginine has poor bio-availability that is why oral L-arginine supplementation does not increase blood levels of arginine. Since oral citrulline has high bioavailability, the best way to increase serum arginine levels is by oral citrulline supplementation. **Oral supplementation of L-citrulline in preterm infants once they are off TPN will likely to increase arginine levels and NO production.**

Safety of oral citrulline: L-citrulline has been safely used for decades in patients with urea cycle defects. It has been used in pediatric patients with sickle cell disease and in infants undergoing cardiac surgery. No side effects were reported in these studies. In a study in newborn rats exposed to hyperoxia, L-citrulline caused a marked increase in arginase-2 expression in the lungs and this could have an impact on lung development and remodeling(5). However, this is only a theoretical risk.

Oral L-citrulline supplementation may prevent and/or decrease the severity of BPD_PH. Since oral L-citrulline supplementation has never been studied in preterm infants before, the side effect profile and appropriate dosing is still unknown. **In this pilot study, we will determine the safety profile, efficacy and appropriate dosing of oral L-citrulline in preterm infants.** In future, information from this study will be utilized to conduct a randomized placebo-controlled trial to evaluate the role of L-citrulline supplementation in treating BPD_PH.

2.0 Rationale and Specific Aims

Specific Aim 1 -

To test the hypothesis that enteral supplementation of L-citrulline (a precursor of L-arginine) is effective in increasing plasma L-arginine and L-citrulline concentrations in premature infants with increased NO production.

Intervention: Enteral L-citrulline supplementation

Rationale & feasibility - L-arginine is a precursor of NO production. Premature infants who develop BPD_PH have lower levels of L-arginine and L-citrulline, decreased NO production, eventually leading to the development of pulmonary hypertension. Oral arginine is poorly bioavailable hence oral L-citrulline will be used (precursor of L-arginine, see figure below). We expect L-citrulline supplementation in premature infants will increase plasma L-arginine and L-citrulline concentrations and increase NO production (measured indirectly by urinary end-products).

Specific Aim 2 -

To determine the safety of oral L-citrulline supplementation in premature infants. Increased production of NO may lead to systemic hypotension by its vasodilator effect. All infants in the neonatal ICU are always on electronic monitors and monitored for heart rate, oxygen saturations and BP. We will continuously monitor all infants for systemic hypotension. Significant hypotension will be defined as a 25% drop in systemic mean blood pressure (measure by non-invasive blood pressure monitoring). Other parameters to be recorded include increase in oxygen requirements/escalation in respiratory support, metabolic abnormalities (on routine glucoses and electrolyte monitoring), effect on bone-marrow by weekly complete blood counts (done routinely on preterm infants).

Specific Aim 3 –

To determine the dose of enteral L-citrulline required to increase plasma level of > 37 $\mu\text{mol/L}$.

We will use three doses (low, medium and high) of enteral L-citrulline as follows -

Group 1: Low dose 50 mg/kg given two times a day (100 mg/kg/day) for total 7 days.

Group 2: Medium dose 100 mg/kg given two times a day (200 mg/kg/day) for total 7 days

Group 3: High dose 150 mg/kg given two times a day (300 mg/kg/day) for total 7 days

Rationale:

Previous studies in patients undergoing cardiac surgery have shown that post-operative pulmonary hypertension did not develop in patients with citrulline level $> 37 \mu\text{mol/L}$ (7). A cross-sectional study reported that citrulline levels $< 29 \mu\text{mol/L}$ was associated with BPD_PH (100% sensitivity and 75% specificity) hence this may be used as a screening tool for BPD_PH(3). We will use the higher levels of citrulline of the reported levels as our goal ($>37\mu\text{mol/L}$). We will supplement infants with 3 different dosages and check serum levels to see what dose is required for to achieve optimal plasma level of citrulline ($>37\mu\text{mol/L}$).

3.0 Inclusion/Exclusion Criteria

Inclusion criteria - Subjects must meet all of the inclusion criteria in order to be eligible to participate in the study.

1. Infants ≤ 30 weeks' gestational age born at UTMB, Galveston.
2. Parents have provided informed consent/assent in a manner that is approved by the IRB

The reason for the inclusion of such a vulnerable population is that BPD is primarily a disease of premature infants and PH is a common complication of BPD. It is also a condition with high mortality and there still exists significant confusion over appropriate management. Being a randomized controlled trial, the study does add certain risks to the subjects: frequent blood draws (minimized by using discarded blood and urine samples) . Since diagnosis and management of BPD_PH is not currently standard of practice, we will not be withholding any beneficial therapy. The present research protocol will not interfere in the management of the preterm infants as treating neonatal team will be taking all management decisions. All risks will be explained to the parents and written informed consent will be obtained from the parents of all subjects.

Exclusion criteria - Subjects meeting any of the exclusion criteria at baseline will be excluded from study participation.

1. Known congenital or chromosomal anomalies.
2. Congenital heart disease affecting cardio-respiratory system (other than PDA, PFO or ASD)
3. NEC, sepsis, or any condition requiring surgery prior to recruitment

4.0 Treatment Assignment/Randomization

Recruitment - Parents of all eligible infants will be approached by research investigator to discuss about the study & research protocol in detail. All questions & concerns will be answered in very simple language & parents will be given written consent form to read. Within 24 to 48 hours, the parents will be approached again to discuss & explain any question they have. We expect around 70 - 75% success in the recruitment. Every year, we deliver around 50 infants (≤ 30 weeks' gestation). We hope to recruit our desired sample of 42 infants (14 in each study group) within 12 months.

Sample size: 14 preterm infants will be randomized to each group (total sample 42) A sample size of 14 at each group (low, median, and high dose) for efficacy endpoint will reach a power of 0.80 to detect an effect size of 0.5 based on one-way ANOVA with a 0.05 significance level. A sample size of 11 at each group for safety endpoint will reach a power of 0.92 at a 0.05 significant level for an equivalence test of means using two one-sided tests, when the true difference between the means is 0.0 mmHg, the standard deviation is 10.0 mmHg, and the equivalence limits are -15.0 mm Hg and 15.0 mmHg.

Randomization: Adaptive randomization method will be used – each new participant will be sequentially assigned to a particular treatment group by taking into account the previous assignments of participants. For example, first recruit will be Group 1, second Group 2, third Group 3, fourth Group 1 and so on. The study will be blinded to the nurses and parents. If patients complete 12/14 doses of the treatment, they will be analyzed in the treatment arm.

Study groups –

Group 1: Low dose 50 mg/kg given two times a day for a total of 7 days (100 mg/kg/day)

Group 2: Medium dose 100 mg/kg given two times a day for a total of 7 days (200 mg/kg/day)

Group 3: High dose 150 mg/kg given two times a day for a total of 7 days (300 mg/kg/day)

Justification for dosing: Pharmacokinetic studies show that half-life of IV citrulline is 60 minutes(8). Half-life of enteral Citrulline has not been studied but previous studies by Montgomery et al, Schwedhelm et al and Waugh et al reported efficacy with twice daily dosing(3, 6, 9). We will also use twice daily dosing. The actual dosing used in previous studies were 80 mg/kg twice daily and 100 mg/kg twice daily respectively. Study in newborn piglets shows that higher doses are associated with greater efficacy. We will use three different dose ranges to determine efficacy (i.e. plasma citrulline level $> 37 \mu\text{mol/L}$. increase in plasma arginine levels and urinary nitrites/nitrates).

5.0 Study Procedures

Data collection - Antenatal, delivery & post-natal (after birth), all detailed data will be collected from electronic medical records (EMR) by the investigators & saved in a password protected computer. We will calculate SNAPPE 2 (Score for Neonatal Acute Physiology – Perinatal Extension 2 – a 9 point validated scoring system that predicts neonatal mortality(10)) for all subjects to ensure equal distribution of sick infants.

Study drug – Oral/enteral L-Citrulline as 10 % solution (100 mg/ml) will be provided to the bedside nurse by the Investigational Pediatric Pharmacy. The drug will be given via gavage feeding by bolus infusions followed by a 0.5 ml water flush twice daily (1200 and 0000). Bolus dosing will be needed due to the small volumes (0.5–1.5 ml per dose in most infants). The volume of nasogastric tubing used in preterm infants (Ameritus 4.0 Fr 50 cm) is 0.48 ml, therefore we will follow the administration with 0.5 ml of saline/water flush to ensure all the study drug is delivered to the patient.

Dispensing study drug - After enrollment & randomization, the research nurse will inform the pharmacy about randomization group. A 10% solution of L-citrulline will be prepared to administer 50 mg/kg, 100 mg/kg and 150 mg/kg doses for Groups 1,2 and 3 respectively. The shelf life of dispensed L-citrulline is 24 hours. L-citrulline will be dispensed as a single dose twice daily for each subject.

Administration of study drug - Will be given via gavage feeding tube twice daily (1200 ± 30 mins, 0000 ± 30 mins). The time of 0000 will be in the window of citrulline's peak serum levels at 5-8 hours when routine blood collection is done at 0400(11). L-citrulline will be given by the bedside nurse as a bolus followed by 0.5 ml water flush. L-citrulline will be given separate from feeds to avoid any confusion.

Dose of study drug –

Low dose 100 mg/kg/day

Medium dose 200 mg/kg/day

High dose 300 mg/kg/day

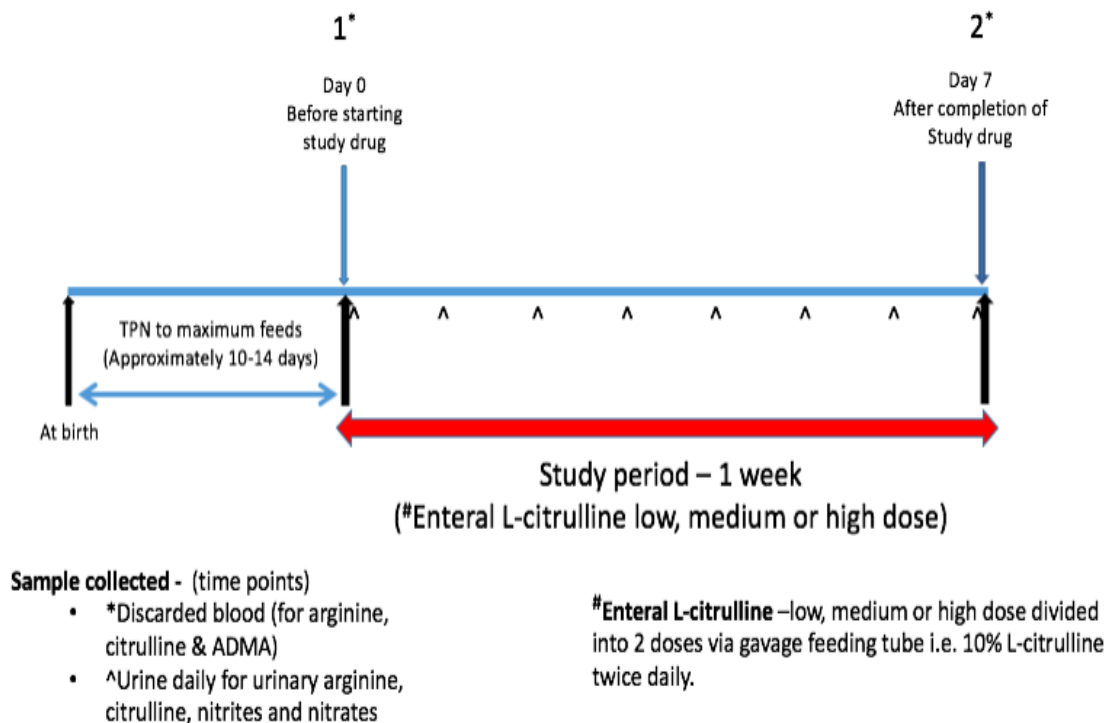
When to start study drug - Study drug will be started when infant has been off of TPN for at least 3 days so that IV arginine in TPN does not interfere.

Sample collection - 1 mL of blood will be collected from each subject at 2 time points: before starting citrulline (Day 0) and then after completing 1 week enteral L-citrulline supplementation (Day 7). Discarded blood sample from routine blood draw will be used.

It is standard of care for all preterm infants to get weekly blood draws on Tuesdays for hemoglobin monitoring. We will use discarded blood sample from the Tuesday after being on full feeds (no TPN) and start enteral L-citrulline supplementation on the first Tuesday (Day 1) after off TPN for at least 3 days. After 1 week, supplementation will be discontinued (last dose Monday PM) and discarded blood sample from Tuesday (Day 7) will be used to analyze plasma levels of L-citrulline and L-arginine. Immediately after blood collection, blood will be centrifuged at 4-degree C to extract serum. Serum will be divided into aliquots of 100 microliter & stored at -80-degree C for future use.

1 mL of urine will be collected before starting (Day 0) and then daily thereafter till the last day of L-citrulline supplementation (Day 7). Urine will be collected by placing cotton balls in diapers at around noon \pm 2 hours (in between the 2 doses of study drug).

All sample collection will be part of routine/standard care of the infant and no extra blood draws/invasive procedures will be needed.



Measurements:

Serum L-citrulline & L-arginine measurement - We will measure amino acids (AA) in serum using an HPLC-MS approach. A cocktail of stable-isotope enriched AA will be added to serum samples & AA will be extracted using a solid-phase cartridge. Extracted AA will be separated by HPLC (C-18 Colum) eluted with a methanol ammonium formate gradient. AA will be injected directly into a QTRAP 6500 instrument tuned for low mass analyses. AA will be detected using characteristic fragmentation ions. Concentrations of AA in the original sample will be determined by co-paring peak areas to those of the stable isotope-enriched internal standards. For this initial study, we will measure citrulline & arginine.

Urinary nitrates and nitrite measurement (metabolites of NO) - Urinary nitrite, nitrate and creatinine will be measured by GC-MS following conversion to the pentafluorobenzyl derivatives.

6.0 Study Product Description

Information in this section can usually be obtained from the IB or the package insert.

Formulation, Packaging, and Labeling

Product Storage and Stability

Keep container dry. Keep in a cool place. Ground all equipment containing material. Keep container tightly closed. Keep in a cool, well-ventilated place. Combustible materials should be stored away from extreme heat and away from strong oxidizing agents.

Precautions: Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evaporate the residue under a fume hood. Ground all equipment containing material. Do not breathe dust. Wear suitable protective clothing in case of insufficient ventilation, wear suitable respiratory equipment If you feel unwell, seek medical attention and show the label when possible. Avoid contact with skin and eyes

Dosage, Preparation and Administration of Study Intervention/Investigational Product

A 10% L-citrulline solution will be prepared using the powdered product by adding 10 g of powder in 100 ml of distilled water (each ml = 100 mg). Doses prepared will be determined by the study group assignment. For e.g., for a 2 Kg infant in Group 1 (50 mg/kg dose = 1 ml). Doses will be administered twice daily.

Safety precautions: Gloves Splash goggles, lab coats, dust respirators and gloves should be used while preparing the doses. Avoid direct contact with skin. Prepared solutions can be held in a cool place for a maximum of 24 hours.

Modification of Study Intervention/Investigational Product for a Participant

No dose modifications will be done for specific lab values. In case of toxicity, study will be aborted as discussed in section 7.0 below.

Accountability Procedures for the Study Intervention/Investigational Product(s)

Study drug (L-citrulline) will be bought from the vendor (VitaFlo) by the PI. The product will be shipped at one time. About 300 g of product will be needed for this study. Unused product will be in the investigational pharmacy for future studies.

Assessment of Subject Compliance with Study Intervention/Investigational Product

Since all doses will directly be administered by the bedside nurse, non-compliance is not applicable to this study. Subjects must receive 12/14 doses to be considered a complete subject.

Concomitant Medications/Treatments

There will be no medication or treatment restrictions during this study. All premature infants will continue to receive standard of care treatment.

7.0 Adverse Events or Unanticipated Problems Involving Risk to Participants or Others

Immediate risks: Increased NO production may theoretically lead to systemic hypotension – all these infants will be in an intensive care setting with cardiac monitors and continuous blood pressure monitoring. Significant hypotension will be defined as a 25% drop in systemic mean blood pressure from baseline (average mean arterial pressure in the week prior to starting study drug).

Local irritation to skin or eyes is possible with direct contact with study drug.

Long-range risks: A theoretical risk of adverse impact on structural remodeling of pulmonary architecture vis increase in arginase two expression in lungs (seen in rats).

Rationale:

Children with urea cycle defects have been treated with L-citrulline for decades and showed no evidence of toxicity. Previous studies in literature have not reported any significant adverse effects. The benefit of treatment (helping treat or prevent BPD_PH) far outweighs the risks since this condition is associated with significant morbidity/mortality and no preventative measures exist at this time. The study drug has already been tested on animal models and there are no alternatives to studying the safety, efficacy and dosing in preterm infants.

ICH E6 defines an AE as any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product regardless of its causal relationship to the study treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of medicinal (investigational) product.

All AEs including local and systemic reactions not meeting the criteria for “serious adverse events” should be captured on the appropriate CRF. Information to be collected includes event description, time of onset, clinician’s assessment of severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis, which would include MD, PA, Nurse Practitioner, DO, or DDS), and time of resolution/stabilization of the event. All AEs occurring while on study must be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution.

Any medical condition that is present at the time that the patient is screened will be considered as baseline and will not be reported as an AE. However, if it deteriorates at any time during the study, it should be recorded as an AE.

All AEs must be graded for severity and relationship to study product.

Severity of Event: All AEs will be assessed by the clinician using a protocol defined grading system. For events not included in the protocol defined grading system, then the following guidelines will be used to quantify intensity.

- Mild: events require minimal or no treatment and do not interfere with the patient’s daily activities.
- Moderate: events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.

- Severe: events interrupt a patient's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually incapacitating.
- Life threatening: any adverse drug experience that places the patient or subject, in the view of the investigator, at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that had it occurred in a more severe form, might have caused death.

Changes in the severity of an AE should be documented to allow an assessment of the duration of the event at each level of intensity to be performed. Adverse events characterized as intermittent require documentation of onset and duration of each episode.

Protocol Grading System for Hypotension:

Mild: Drop in systemic mean arterial pressure (MAP) \leq 10% from baseline

Moderate: Drop in systemic mean arterial pressure (MAP) \leq 25% from baseline

Severe: Drop in mean arterial pressure (MAP) $>$ 25% from baseline

Life threatening: Severe hypotension requiring 2 or more inotropes (Dopamine, Epinephrine, Norepinephrine)

Relationship to Study Products: If there is any doubt as to whether a clinical observation is an AE, the event will be reported. All AEs must have their relationship to study product assessed using the terms: associated or not associated. In a clinical trial, the study product must always be suspect. To help assess, the following guidelines are used.

- Associated – The event is temporally related to the administration of the study product and no other etiology explains the event.
- Not Associated – The event is temporally independent of study product and/or the event appears to be explained by another etiology.

Serious Adverse Event (SAE): An SAE in our study will be defined as an AE that meets one of the following conditions:

- Death during the period of protocol defined surveillance
- Life-threatening event (defined as a subject at immediate risk of death at the time of the event) – significant hypotension requiring 2 or more inotropes

- Results in a persistent or significant disability/incapacity: Intraventricular hemorrhage Grade III or more, Necrotizing enterocolitis requiring surgery, Retinopathy of prematurity requiring surgery
- Significant lab abnormalities: Thrombocytopenia < 50,000

All SAEs will be:

- recorded on the appropriate SAE CRF
- followed through resolution by a study clinician
- reviewed and evaluated by a study clinician

A DSMB (data safety monitor board). DSMB will review data (from CRFs) every 3 months to make sure it is safe to give citrulline to preterm infants.

DSMB will have -

1. Dr. Joan Richardson MD, Neonatologist, Prof and Chief of Neonatal Division, UTMB
2. Dr. Ashraf Aly MD, PhD, Pediatric Cardiologist, Prof Department of Pediatrics, UTMB
3. Dr. Maged Costantine MD, Maternal Fetal Medicine, Associate Prof, Department of ObGyn, UTMB
4. Dr. Karen Smith PhD, Pediatric Psychologist, Prof Department of Pediatrics

Procedures to be Followed in the Event of Abnormal Laboratory Test Values or Abnormal Clinical Findings

Reporting Procedures

Discarded blood samples from routine blood draws will be used for study measurements. No extra blood will be drawn from infants. This study does not involve any invasive procedures.

Any AE considered serious by the PI or Sub investigator or which meets the aforementioned criteria must be submitted on the SAE form to the IRB in accordance with IRB policies and procedures.

The study clinician will complete a Reportable Event Form within the following timelines:

- All serious adverse events described above to the UTMB IRB will be reported via InfoED utilizing the Reportable Event Form within 10 days.
- Serious adverse events involving an unexpected death or life-threatening experience related to research which be reported to the UTMB IRB within 2 days after first knowledge by the principal investigator.

All SAEs will be followed until satisfactory resolution or until the PI or Sub investigator deems the event to be chronic or the patient to be stable.

8.0 Study Withdrawal/Discontinuation

Reasons for Withdrawal

A study subject will be discontinued from participation in the study if any of the following occur:

- Unacceptable toxicity or adverse event (AE): Drop in systemic mean blood pressure > 25% from baseline
- Unacceptable laboratory abnormalities: Thrombocytopenia < 50,000 not explained by other co-existing conditions
- Intercurrent illness, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the subject in the opinion of the treating investigator, for e.g. necrotizing enterocolitis requiring subject to be made NPO.
- Development of any exclusion criteria
- Parents withdraw consent to continue in the research for any reason

Handling of Withdrawals

Any subjects that withdraw from the study will continue to receive standard of care treatment according to our NICU protocols and will continue to be followed clinically by the primary neonatologist. Safety data from these subjects will be presented to the DSMB for evaluation.

Since this is a pilot study, we will not need to replace subjects in case of withdrawals.

Termination of Study

Given the short duration of the trial, we do not plan to conduct any interim analyses or specify any early-stopping rules.

9.0 Statistical Considerations

Study design: Single-center Pilot dose-ranging study

Study population: Premature infants admitted to the Neonatal intensive care unit at UTMB, Galveston.

Rationale: L-citrulline has been shown to increase NO production in neonatal pigs. The use of L-citrulline in premature infants may help increase NO and prevent pulmonary hypertension that develops due to chronic lung disease. Even though L-citrulline has been used safely in infants and children for urea cycle disorders, its dosage, efficacy and safety have not been evaluated in premature infants. Therefore, a pilot dose-ranging study is essential.

There will be 3 study groups in this Phase I trial:

Group 1 (Low dose): 100 mg/kg/day of L-citrulline

Group 2 (Medium dose): 200 mg/kg/day of L-citrulline

Group 3 (High dose): 300 mg/kg/day of L-citrulline

Sample size: A sample size of 14 at each group (low, median, and high dose) for efficacy endpoint will reach a power of 0.80 to

detect an effect size of 0.5 based on one-way analysis of variance (ANOVA) with a 0.05 significance level. The sample size calculation was based on the following expected mean \pm SD citrulline concentrations in plasma according to the study from Schwedhelm et al:

Low dose group: 19 ± 4 μ mol/L

Medium dose group: 21 ± 4 μ mol/L

High dose group: 45 ± 5 μ mol/L

Primary outcome:

1. Increase in plasma L-citrulline levels and increased NO production (measured indirectly via urinary breakdown products)

Secondary outcomes:

1. To determine safety of enteral L-citrulline supplementation in premature infants.
2. To determine the dose of enteral L-citrulline needed to increase plasma level of citrulline > 37 mol/L.

Data will be collected from electronic medical records. Measurements will be recorded in a password protected computer on an excel sheet.

A Data Safety Monitor Board (DSMB) as mentioned in section 7.0 will be reviewing safety data every 4 months.

The data will be analyzed at the end of data collection for all subjects who meet the inclusion criteria. SPSS version 24 will be used for data analysis. SAS procedures, linear models, repeated measures analysis of variance (ANOVA) & logistic modeling for receiver operator curve generation (significance $p < 0.05$) will be used. The UTMB Clinical trials studio will be involved to assist in statistics.

Procedure for accounting for any missing, unused and spurious data:

In this pilot study, subjects with missing data on important predictors (lack of blood sample for serum arginine/citrulline levels) will be excluded from analyses.

Selection of subjects to be included in the analyses:

We will follow an intention-to-treat approach in the analyses. The primary variables (plasma arginine, citrulline levels and urinary nitrite, nitrate levels) are continuous data

and the one-way ANOVA will be used to analyze differences amongst the 3 treatment groups from day 0 and day 7. Paired T-test will be used to evaluate differences pre-and post-treatment in individual subjects. The data gathered from daily urinary nitrite and nitrate measurements will be represented in line graphs to represent the trend.

10.0 Ethics and the Protection of Human Subjects

The investigator will ensure that this study is conducted in full conformity with the principles set forth in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research of the US National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research (April 18, 1979) and codified in 45 CFR Part 46 and/or the ICH E6; 62 Federal Regulations 25691 (1997).

The investigator must provide for the review and approval of this protocol and the associated informed consent/parental assent documents and recruitment material by an appropriate independent ethics committee (IEC) or IRB registered with the OHRP. Any amendments to the protocol or consent materials must also be approved before they are placed into use. In the United States and in other countries, only institutions holding a current US Federalwide Assurance issued by OHRP may participate.

Informed consent is a process that is initiated prior to the individual's (in this case, the legal guardians) agreeing to participate in the study and continuing throughout the individual's study participation. Extensive discussion of risks and possible benefits of this therapy will be provided to the parents/legal guardians of the study subjects. Assent forms describing in detail the study interventions/products, study procedures, and risks are given to the subject and written documentation of informed consent is required prior to starting intervention/administering study product. Assent forms will be IRB-approved and the parents will be asked to read and review the document. Upon reviewing the document, the investigator will explain the research study to the parents and answer any questions that may arise. The parents will sign the informed assent document prior to any procedures being done specifically for the study. The parents should have the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. The parents may withdraw consent at any time throughout the course of the trial. A copy of the informed consent document will be given to the subjects for their records. The rights and welfare of the subjects will be

protected by emphasizing to the parents that the quality of their medical care will not be adversely affected if they decline to participate in this study.

Privacy/Confidentiality Issues

Subject confidentiality is strictly held in trust by the participating investigators, their staff, and the sponsor(s) and their agents. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participating subjects.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the Principal investigator.

Individuals authorized to view study records will be identified to the study subject on the informed consent but may include a study monitor or other authorized representatives of the sponsor or funding agency or federal or local agencies that regulate research including representatives from the Food and Drug Administration, Office of Human Research Protections, or the Institutional Review Board. Study records may also be made available for internal compliance reviews and quality assurance representatives.

These individuals may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the subjects in this study. The clinical study site will permit access to such records.

In accordance with the Food and Drug Administration Amendment Act of 2007 (FDAAA) and The International Committee of Medical Journal Editors (ICMJE) member journals trials-registration policy as a condition for publication, this study will be registered in the public trials registry [ClinicalTrials.gov](https://clinicaltrials.gov), which is sponsored by the National Library of Medicine. Results will be published to [clinicalTrial.gov](https://clinicaltrials.gov) when available but will not identify individual subjects.

11.0 Record Retention

The expected duration of this study is 1 year,

Since this research study accesses protected health information (PHI) and is covered under the Health Insurance Portability Accountability Act (HIPAA), consent forms that

include the HIPAA Authorization are to be retained for a minimum of 6 years from the date of the authorization.

Since this Research involves pediatric subjects, records shall be maintained until the last participating subject reaches age 21 or 10 years after completion of the research, whichever is later.

Research records will be maintained in accordance with the current version of the Texas Health Records Table 17-III Record Retention Schedule, Human Subject Research Records and Documents.

12.0 References

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