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Effect of High-Dose Cysteine Supplementation on Erythrocyte Glutathione: a Double-Blinded, Randomized Placebo Controlled Pilot Study in Critically Ill Neonates

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Abstract

Background—This study's objective was to determine if parenteral cysteine when compared to isonitrogenous non-cysteine supplementation increases erythrocyte reduced glutathione (GSH) in neonates at high risk for inflammatory injury.

Material and Methods—Neonates with a score for neonatal acute physiology (SNAP) > 10 requiring mechanical ventilation and parenteral nutrition (PN) were randomized in a double-blinded, placebo controlled study to receive parenteral cysteine-HCl (CYS group) or additional PN amino acids (ISO group) at 121 mg/kg/day for seven days. A six-hour [¹³C₂] glycine IV infusion was administered at study week one to determine fractional synthetic rate of glutathione (FSR-GSH).

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Conflicts of Interest:

The authors have no conflicts of interest to disclose.

Results—Baseline characteristics were similar between the CYS (n=17) and ISO groups (n=21). Erythrocyte GSH and total glutathione concentrations, GSH:oxidized glutathione (GSSG), and FSR-GSH after treatment were not different between groups. However, the CYS group had a larger individual positive change in GSH and total glutathione (infusion day – baseline) compared to the ISO group ($p=0.02$ for each). After adjusting for treatment, a lower enrollment weight and red blood cell (RBC) transfusion were associated with a decreased change in total glutathione and GSH ($p<0.05$ for each).

Conclusion—When compared to isonitrogenous non-cysteine supplementation, high dose cysteine supplementation for at least one week in critically ill neonates resulted in a larger and more positive individual change in glutathione. Smaller infants and those who received transfused blood demonstrated less effective change in glutathione with cysteine supplementation. The benefit of cysteine remains promising and deserves further investigation.

Keywords

neonates; parenteral nutrition; antioxidant; inflammation; red blood cell; prematurity; chronic lung disease

Introduction

Critically ill neonates lack adequate antioxidant defense systems and are at high risk for oxidative and inflammatory injury exhibited by respiratory distress syndrome, chronic lung disease, retinopathy of prematurity, necrotizing enterocolitis, sepsis, and perinatal brain injury—all of which are associated with a high morbidity, mortality, and healthcare cost.^{1–3} A common pathway connecting these diseases is the imbalance between oxidative and inflammatory stress and endogenous antioxidant defenses. Oxygen free radicals indiscriminately react with cell membranes causing lipid peroxidation, cell proteins resulting in protein degradation, and DNA causing mutations and cell death. Hypoxic-ischemia reperfusion injury (associated with necrotizing enterocolitis and perinatal brain injury) and prolonged exposure to a high fractional inspired oxygen concentrations and sepsis (associated with chronic lung disease) result in oxygen free radicals and systemic inflammation—all of which are major risk factors for adverse neurodevelopmental outcomes and other common long-term neonatal comorbidities.^{4–6}

Cells are equipped with various antioxidant defenses to protect against free radicals and inflammation. These include catalytic enzymes such as catalase, superoxide dismutase, glutathione peroxidase, as well as noncatalytic reducing substances such as vitamin E, vitamin C, vitamin A, and reduced glutathione (GSH). Glutathione is a promising antioxidant to help fight against free radicals and inflammation—it is the body's most abundant antioxidant and present in most cells. GSH is an intracellular tripeptide synthesized from cysteine, glutamate and glycine and can be regenerated from the oxidized form of glutathione (GSSG) via glutathione reductase. GSH provides cellular protection by conjugating with lipid peroxidation products via glutathione-S-transferases and scavenging free radicals via glutathione-peroxidase (Figure 1).

Prior studies in animals, adults, and children have demonstrated that cysteine supplementation increases glutathione concentrations and synthetic rates and decreases levels of certain biological markers.^{7–11} In preterm neonates, cysteine is considered by some investigators to be “conditionally essential”—an amino acid that requires an exogenous supply during specific time periods due to decreased supply, increased GSH demand, and/or decreased ability to synthesize or regenerate GSH.^{12,13} Since critically ill neonates are often dependent on parenteral nutrition (PN), providing cysteine maybe the most facile way to increase glutathione stores and availability, decrease markers of oxidative injury and inflammation, and neonatal disease burden.

Subsequently, parenteral cysteine supplementation has become common in most neonatal intensive care units in the United States. However, current data for this population remains inconclusive for a variety of reasons^{11,14–20}. Different studies have provided cysteine at various doses, in different forms, and have mainly concentrated on low birth weight neonates. Primary outcomes have also been different (cysteine vs. glutathione), and some outcomes were measured soon after birth.^{11,14–20} Many studies have measured total glutathione, but not GSH that would have more accurately reflected a cell’s redox potential.^{11,14–20} The risk for inflammatory and oxidative injury may persist and even increase throughout a neonate’s hospital course, making cysteine supplementation beyond the first week of life potentially beneficial, and has not been adequately addressed in prior studies. Lastly, full-term neonates, like their preterm counterparts, are at high risk for oxidative/inflammatory injury.² As a result, it remains unclear what the optimal dose and duration of cysteine is and whether populations other than preterm infants may benefit from cysteine supplementation. The purpose of this pilot study is to determine if a relatively high dose of cysteine supplementation for at least 1 week within the first month of life increases intracellular GSH concentrations in PN-dependent critically ill neonates.

Methods

Study Outcomes

All glutathione concentrations were intracellular erythrocyte concentrations unless otherwise noted. The primary outcome was GSH concentration. Secondary outcomes included oxidized glutathione (GSSG) and total glutathione concentration, GSH:GSSG, fractional synthetic rate of glutathione (FSR-GSH), plasma interleukin-6 (IL-6) concentration, and days in the hospital and requiring supplemental oxygen or ventilation. Chronic lung disease (need for supplemental oxygen after 36 weeks postmenstrual age if born < 34 weeks gestation or for one month for neonates born ≥ 34 weeks gestation), necrotizing enterocolitis, sepsis (a culture confirmed bloodstream infection after 72 hours of life), intraventricular hemorrhage grade III and IV, and retinopathy of prematurity requiring laser treatment were recorded as tertiary outcomes.

Power analysis (assuming: $\beta = 0.2$, $\alpha = 0.05$) using prior published data to detect a clinically significant difference of 20% in the primary outcome determined that a sample size of 36 infants per arm in three weight strata was required to reject the null hypothesis.²¹

Patient Population

Inclusion criteria for this single center study were neonates requiring mechanical ventilation who were < 30 days of life with a score for neonatal acute physiology (SNAP) > 10 and projected PN requirement seven days. Exclusion criteria included renal or hepatic failure, insulin requirement, extracorporeal membrane oxygenation, or a known inborn error of metabolism. Informed, written consent was obtained from parents/legal guardians. The study was approved by the University of California, Los Angeles Institutional Review Board and registered with www.clinicaltrials.gov (NCT00254176).

Study Procedures and Methods

Subjects were randomized in blocks of six to receive either cysteine (CYS group) or isonitrogenous non-cysteine supplementation (ISO group) and stratified by birth weight (1499 g, 1500–2499 g, 2500 g). The investigational pharmacy allocated the assigned supplement masked to the medical team and investigators. All subjects received PN comprised of dextrose, amino acids (10% Premasol®, Baxter, Deerfield, IL - containing minimal cysteine-HCl of 0.016 g/100 mL), and lipids (20% Intralipid®, Fresenius Kabi, Uppsala, Sweden) along with 121 mg/kg/day of supplement (approximately 40 mg/g-AA/day) as either: cysteine-HCl (Sicor Pharmaceuticals, Inc, Irvine, CA) and sodium acetate (2 mEq/kg/day to offset the additional acid load), or additional Premasol® amino acids.²² The supplement was continued until PN discontinuation or 60 days of PN, whichever came first.

The medical team unrelated to the study prescribed PN. According to common practice in this study's neonatal intensive care unit, PN was prescribed for critically ill neonates within the first 24 hours of life. Goal calories and amino acid dose for term and low birth weight neonates were approximately 100 and 120 kcal/kg/day and 3 g/kg/day and 3–4 g/kg/day, respectively.

Only subjects who received at least seven days of study PN and completed the isotope infusion protocol were included in the analysis. Blood was collected prior to initiation of assigned supplement for baseline concentrations. On approximately study day seven, a primed (35 µmol/kg), continuous (35 µmol/kg/hr) intravenous infusion of [¹³C₂]glycine was administered for six hours to measure outcome concentrations and FSR-GSH similar to previous studies.^{16,19,23} One blood sample (500 µL–1 mL) was collected prior to start of the isotope infusion. After steady state was obtained at four hours as outlined previously, two to three additional blood samples (500 µL–1 mL each) were collected.^{16,19,23} Samples were collected at infusion hours four, five, and six if the subject weighed ≥ 1500 g. If the subject weighed < 1500 g, blood was collected at hours 4 and 6 only because the amount of blood that can be collected in very-low-birth weight neonates is limited due to their size and high risk for anemia. As part of a secondary study, a repeat infusion study was performed if the subject remained on PN at 60 days of life (data not shown). All infusions were delayed by approximately 24 hours if the subject required a red blood cell (RBC) transfusion. Sources of blood sampling were arterial (preferred), venous, or warmed-heal stick. Samples were collected in pre-chilled ethylenediaminetetraacetic acid (EDTA) tubes, centrifuged to separate plasma from RBCs, and immediately stored at –80°C until laboratory analysis was performed.

Intracellular concentrations of GSH, GSSG, cysteine, glycine, glutamate and isotope ratios of [$^{13}\text{C}_2$]glycine incorporation into GSH were determined by using liquid chromatography-tandem mass spectrometry. A mixture of deuterated internal standards were added to weighed, volume-measured (100 μL) sample RBCs that were then treated with methanol/100 mM ammonium bicarbonate and 4-vinylpyridine (30°C, 30–160 minutes) then dried in a vacuum centrifuge. Samples were then treated with methanolic HCl (3N, 100 μL , 60°C/60 minutes) and dried again, re-dissolved in water (100 μL) and injected (5 μL) onto a reverse phase high performance liquid chromatography column (Phenomenex Kinetex XB-C18, 100 $\times$ 2.1mm, 1.7 μm particle size, 100 Å pore diameter; Torrance, CA) utilizing 1mM perfluorooctanoic acid in H_2O and acetonitrile buffers with the effluent directed to an electrospray ion source (Agilent Jet Steam, Wilmington, DE) connected to a triple quadrupole mass spectrometer (Agilent 6460, Wilmington, DE) operating in the tandem mass spectrometric multiple reaction monitoring mode. Recordings were made of select parent to fragment ion transitions for the metabolites of interest using previously optimized conditions.²⁴ Sample concentrations were determined by interpolation from standard curves prepared with each batch of samples using peak area measurements. Isotopic enrichment was measured from areas under the curve for respective ^{13}C (i.e., M+2) and ^{12}C (M) molecules. Isotopic ratios expressed as Mol % Excess (MPE) over background enrichment were then calculated based on standard equations.²⁴ The FSR-GSH was calculated in %/day using the following equation:

$$[\text{GSHglyc}(t_6) - \text{GSHglyc}(t_4)]/\text{RBCglyc} \times 1200$$

where $\text{GSHglyc}(tx)$ is the isotope ratio of the GSH-bound glycine at the fourth and sixth hour of the [$^{13}\text{C}_2$]glycine infusion, RBCglyc is the mean isotope ratio for the free intracellular glycine within the erythrocyte at steady state, and 1200 is the time constant to convert the result to %/day. Plasma IL-6 concentrations were determined using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN).

Statistical Methods and Analysis

Continuous variables were compared using the Mann-Whitney U-test and categorical variables were compared using the Fisher's exact test. Linear regression was used to examine covariates and their interaction with the group assignment. A p-value of < 0.05 was considered statistically significant. No adjustments were made for multiple comparisons. R version 2.15 statistical software was used (www.r-project.com).

Results

Between November 2006 and October 2010, 46 subjects were enrolled, and 38 subjects (CYS = 21, ISO = 17) completed the study. The study was discontinued for slow recruitment after the preliminary analysis. Reasons for subjects not completing the study included death unrelated to study intervention (1), withdrawal because of renal failure unrelated to study intervention (2), transfer to an outside hospital (3), discontinuation of PN prior to the stable isotope infusion (1), and lack of prepared [$^{13}\text{C}_2$]glycine by investigational pharmacy (1). Baseline characteristics between the two groups were comparable (Table 1).

Both groups on average were premature with a gestational age ($\pm$ SD) of 34 ± 6 weeks ($p=0.6$). The admission diagnoses in the CYS group and ISO group were similar: 33% and 35% had respiratory distress syndrome, 18% and 14% had persistent pulmonary hypertension (6 out of 7 subjects required inhaled nitric oxide), 33% and 24% had congenital heart disease (hypoplastic heart (4), transposition of the great arteries (3), tetralogy of fallot (3), double outlet right ventricle (1)), and 14% and 12% had other diagnoses that were mainly congenital gastrointestinal disorders such as gastroschisis ($p=0.9$).

At the day of the infusion, PN study supplement days were higher in the CYS group when compared to the ISO group with a mean ($\pm$ SD) of 9 ± 0.7 vs. 7 ± 3 days ($p=0.002$). The two groups were similar with regards to total PN calories (kcal/kg/day), AA dose (g/kg/day), and percent of subjects who received partial enteral feeds prior to the infusion study day (Table 1). Enteral nutrition was minimal in both groups with a median (range) enteral intake of 1.2 (0.6–5.6, $n=4$) and 1.3 cc/kg/hour (0.1–0.9, $n=3$) in the CYS group and ISO group respectively ($p=0.9$). When the CYS group was compared to the ISO group, the anion gap at baseline was comparable (8 ± 2 vs. 9 ± 3 , $p=0.5$). However, the CYS group had a higher mean anion gap post supplement on infusion day (12 ± 3 vs. 9 ± 4 , $p=0.01$) than the ISO group (Table 1).

At infusion day, no difference was measured for intracellular cysteine (91 ± 86 vs. 75 ± 78 μ mol/L, $p=0.3$), glycine (386 ± 311 vs. 389 ± 275 μ mol/L, $p=0.5$), and glutamate concentrations (454 ± 220 vs. 438 ± 189 μ mol/L, $p=0.8$) in the CYS group compared to the ISO group. The CYS and ISO group means were not different for total glutathione, GSH, and GSH:GSSG, neither at enrollment nor infusion day (Table 2). The mean ($\pm$ SEM) MPE of intracellular [$^{13}\text{C}_2$]glycine during steady state between infusion hours four and six was 5.7 ± 0.5 (coefficient of variation: $13\pm 2\%$) and 5.7 ± 0.5 (coefficient of variation: $12\pm 1\%$) for the CYS and ISO groups, respectively, and were not different between groups ($p=0.6$). The MPE of [$^{13}\text{C}_2$]glycine incorporation into GSH (0.21 ± 0.02) from infusion hour four to six was at appropriately detectable levels for all subjects and similar to other studies.^{16,19,23} The mean glutathione FSR-GSH ($\pm$ SEM) was similar between the CYS group and ISO group (44 ± 5 vs. 49 ± 4 %/day, $p=0.4$) (Table 2). However, the mean individual change ($\pm$ SEM) in total glutathione and GSH (infusion day – baseline) in the CYS group was significantly higher and positive when compared to the ISO group, which was lower and negative (0.5 ± 0.2 vs. -0.2 ± 0.2 , and 0.4 ± 0.2 vs. -0.2 ± 0.2 mmol/L, $p=0.02$ each). While not statistically significant, the mean individual change in GSH:GSSG in the CYS group when compared to the ISO group (-1 ± 2 vs. -4 ± 3 vs mmol/L, $p=0.3$) represented a similar trend (Figure 2). A subgroup analysis of subjects with respiratory distress syndrome, neonates with persistent pulmonary hypertension, and neonates with congenital heart disease did not reveal a difference between the CYS and ISO groups for total glutathione, GSH, and GSH:GSSG.

Analysis of secondary clinical outcomes demonstrated no difference between groups. The mean length of hospital stay was 69 ± 51 and 67 ± 65 days in the CYS group and ISO group, respectively ($p=1$). Mean days requiring supplemental oxygen (44 ± 46 vs. 38 ± 46 days, $p=0.4$) and ventilation (32 ± 35 vs. 32 ± 45 days, $p=0.5$) were similar between the CYS and

ISO groups. Thirty eight percent of the CYS group and 35% of the ISO group developed chronic lung disease ($p=1$). Plasma IL-6 concentrations were not statistically different between the two groups (Table 1).

After adjusting for treatment assignment with a multivariate analysis, lower enrollment weight and the need for a RBC transfusion decreased the change in GSH ($p=0.04$ and 0.03), and RBC transfusion decreased the change in total glutathione ($p=0.03$) (Table 3).

No difference was found in the CYS and ISO groups with regards to the percent of subjects diagnosed with necrotizing enterocolitis (29% vs. 24%, $p=1$), intraventricular hemorrhage grade III and IV (19% vs. 24%, $p=1$), and need for laser treatment for retinopathy of prematurity (24% vs. 24%, $p=1$). No difference was demonstrated in the incidence of late onset sepsis (38% vs. 29%, $p=0.7$). Most subjects with necrotizing enterocolitis were Bell's stage II or III. One subject (5%) in the CYS group and two subjects (12%) in the ISO group expired during the hospital stay ($p=0.6$).

Discussion

In this double-blinded, randomized, placebo-controlled pilot study, cysteine-HCl supplementation in critically ill neonates did not significantly improve erythrocyte GSH:GSSG or FSR-GSH compared to an isonitrogenous non-cysteine supplement despite the use of a relatively high dose (121 mg/kg/d) and treatment period of at least seven days (Table 2). However, cysteine supplementation did result in a positive and significant individual change (infusion day – baseline) in the subject's erythrocyte total glutathione and GSH when compared to the isonitrogenous supplement (Figure 2). Negative results from this trial could be for a variety of reasons. First, cysteine and GSH could have been rapidly oxidized or used for other unmeasured processes, or hepatic GSH stores were adequate to off-set intracellular cysteine deficiencies. In order to decrease the risk for oxidation which could result in artificially elevated GSSG concentrations, samples were treated with the reducing agent, 4-vinylpyridine.²⁵ In addition, while the liver represents the main source of GSH, obtaining hepatic tissue in this study would not have been possible without adding potential morbidity. As a result, we opted to measure intracellular GSH.

The heterogeneous nature of this study population (variation in gestational ages and admission diagnoses) and the smaller than expected sample size that resulted in minor differences between the 2 study groups (enrollment age and duration of cysteine supplementation) may have also limited this study's ability to detect a difference in the primary outcome. However, prior research on cysteine supplementation has been isolated to mainly preterm subjects.^{11,14–20} Full-term infants such as those with cyanotic heart disease, persistent pulmonary hypertension, and congenital gastrointestinal disorders also represent a large population in level III neonatal intensive care units and may benefit from the antioxidant protection offered by potentially increased GSH availability. Approximately 10% of infants who develop necrotizing enterocolitis are full-term and babies with severe persistent pulmonary hypertension are at high risk for chronic lung disease.²⁶ For this reason, the intention of this study was to target high-risk neonates, regardless of gestational age or birth weight. Our results indicate that a higher enrollment weight is independently

associated with an increased, positive individual GSH change (Table 3). This is consistent with our previous work and indicates that the ability to synthesize cysteine and potentially GSH appears to be directly proportional to gestational age.²³ A much larger sample size would be required to adequately address the primary outcome in specific weight strata and could be the focus of future studies. Unfortunately, recruitment for this study was difficult because of the subjects' critical status and blood-sampling requirement.

In contrast to a similar study by Te Braake et al¹⁹, they investigated 10 preterm subjects who received 81 mg/kg/day of cysteine who were compared to 10 subjects who received the standard dose of cysteine (45 mg/kg/day). The subjects' mean birth weight was approximately 1 kg and the median Clinical Risk Index for Babies score was 2 and 3 in the experimental and standard dose group, respectively. The study demonstrated no difference FSR-GSH and plasma cysteine or glutathione on postnatal day two between the two groups. In comparison, in our study, 36 subjects were enrolled with much higher mean birth weight of approximately 2.3 kg.¹⁹ Our subjects may have been sicker as indicated by mean SNAP scores of 14 and 13 and baseline oxygen requirements of 35% and 40% in the CYS group and ISO group, respectively. Moreover, study outcomes were measured after at least 7 days of treatment with 121 mg/kg/day of cysteine.

To target a population at high risk for a suboptimal antioxidant defense system and development of oxidative injury and inflammation, the study's inclusion criteria required the need for mechanical ventilation and a specific SNAP score, a validated tool for assessing severity of critical illness.²⁸ Our study population would be predicted to have a relatively high rate of morbidity and mortality, as evident by not only their gestational age, but also elevated SNAP scores, IL-6 concentrations, need for respiratory support, and presence of co-morbid diagnoses. Although not statistically significant, the CYS group demonstrated a trend toward having higher Apgar and SNAP scores, baseline plasma IL-6 concentrations, and increased need for RBC transfusions, indicating a potentially greater severity of illness for the CYS group at time of enrollment compared with the ISO group despite prospective randomization (Table 1). While our study was originally powered to assess for a 20% change in erythrocyte GSH in each birth weight strata, current sample size may have been sufficient to detect a change in FSR-GSH based off of previously published data in very-low-birth-weight neonates.^{19,23} A sub-group analysis of thirteen very-low-birth-weight neonates, however, failed to demonstrate a difference in GSH, GSH:GSSG, and FSR GSH. It is possible that the sub-group analysis and overall results of the study may have been affected because the CYS group and study population may have been "sicker" compared to the ISO group and previously published cohorts.^{16,19}

A regression analysis of clinical factors contributing to glutathione concentrations demonstrated that the need for a RBC transfusion was independently associated with a decreased change in GSH and total glutathione (Table 3). This factor could have also minimized the impact on glutathione production in the CYS group as there was a perceived higher rate of RBC transfusion in this group (Table 1). Anemia is known to decrease GSH recycling.²⁹ Critically ill and preterm neonates are at high risk for anemia and need for transfusions secondary to lack of enteral nutrition, reduced RBC lifespan, iatrogenic blood losses, and low concentrations of erythropoietin. Processing of donor RBCs negatively

affects glutathione's antioxidant potential, and RBC transfusions have been associated with increased oxidative stress.^{29–31} Measuring GSH in transfused RBCs may be important for future studies to determine the impact that transfusions have on GSH production and inflammatory and oxidative biomarkers. RBC transfusions are also associated with an increased incidence of chronic lung disease, retinopathy of prematurity, and necrotizing enterocolitis.^{32–34} However, it is unclear if this association is a true causal relationship or secondary to the patients' underlying illness. Results from this study may indicate that RBC transfusions may exacerbate oxidative and inflammatory injury by decreasing glutathione stores.

It remains unclear if the results of this study would be altered if cysteine supplementation was started soon after birth and/or the study supplement was continued for more than seven days. While it would be ideal to randomize only inborn subjects soon after birth, the number of eligible subjects would be drastically reduced and this would not have been feasible for this single-site study. Lastly, while parenteral amino acids solutions contain minimal cysteine, they are also devoid of glutamine. Like cysteine, glutamine is a precursor to GSH and has been described to be a conditional essential amino acid in critically ill neonates.^{35,36} In adult studies, parenteral glutamine supplementation has been shown to improve survival and reduce intestinal permeability and sepsis.^{37–39} However, studies in neonates have provided conflicting results.^{35,36,40} Future investigations may consider supplementation with both parenteral cysteine and glutamine to determine the effect on GSH concentrations and specific clinical outcomes.

The factors contributing to changes in glutathione concentrations for critically ill neonates are expected to be multi-factorial. Neonates with respiratory distress syndrome, persistent pulmonary hypertension, and chronic lung disease have been shown previously to have suboptimal plasma and lung glutathione concentrations.^{3,21,41,42} Decreased glutathione concentrations in this population may be a result of: 1) decreased synthesis, 2) increased utilization, or 3) a combination of both. Cysteine, the rate limiting substrate for glutathione synthesis, has historically been labeled by some as a conditionally essential amino acid in premature and critically ill patients.^{12,13} Cystathionine γ lyase and cystathionine β synthase play a vital role in the transsulfuration pathway, which is responsible for synthesizing cysteine from methionine and serine. While the genes for these enzymes are present in the fetal liver, they have been measured to have a variable expression proportional to the extent of prematurity.^{13,43} After birth, the necessary metabolic machinery is induced so that glutathione can be produced. Newborns have higher glutathione umbilical cord blood concentrations in comparison to concentrations measured in adults.^{44,45} This neonatal glutathione reservoir functions as an antioxidant defense as the unborn child transitions from a hypoxic intrauterine environment to a relatively hyperoxic extrauterine environment. During the postnatal period, glutathione concentrations decline as the risk for oxidative injury decreases in a healthy newborn.^{46,47} However, in the critically ill neonate there may be an increased need for substrates to produce appropriate concentrations of antioxidants.

Prior studies investigating the potential benefit of cysteine supplementation in neonates have varied from providing different forms of cysteine supplementation (*i.e.*, cysteine-HCl vs. N-acetylcysteine) and doses for varying treatment periods.^{11,14–20} Study outcomes have also

varied. Some studies have focused on plasma cyst(e)ine while others have focused on different forms of glutathione (total vs. GSH) in different locations (plasma vs. intracellular).^{11,14–20} In a previous double-blinded, randomized controlled trial by Ahola et al¹⁵, premature neonates requiring respiratory support received either 32 mg/kg/day of intravenous N-acetyl-L-cysteine or nonisotonic placebo (i.e., normal saline). There was no difference in chronic lung disease or death.¹⁵ However, N-acetyl-cysteine has been shown to be readily excreted in the urine and is considered to be less bioavailable than cysteine-HCl.⁴⁸ In another study of neonates, PN was supplemented with either 10 mg/kg/day of cysteine-HCl or methionine only. In this study, there was no change in plasma cysteine concentrations or erythrocyte glutathione or glutathione synthetic rates.¹⁷

These studies along with others have led many to believe that adequate provisions of methionine are sufficient for the synthesis of cysteine and glutathione for preterm neonates.^{17,46} Methionine, an essential amino acid, acts as a methyl donor and a substrate for glutathione (Figure 1). In neonates provided with either 2.4 g/kg/day of parenteral amino acids or dextrose solution, those provided with amino acids alone demonstrated an increase in glutathione's absolute synthetic rate.¹⁶ However, when glutathione's synthetic rate was investigated in the amino acid group on study days one and two, glutathione concentrations were increased but without a change in the FSR-GSH.⁴⁹

Cysteine supplementation does have additional benefits beyond possibly increasing glutathione concentrations. Cysteine-HCl reduces the pH of PN increasing the solubility of calcium and phosphorus. As a result, clinicians are able to increase the concentrations of these minerals in PN. Decreased PN administration of calcium and phosphorus concentrations are associated with rickets and neonatal fractures in infants dependent on prolonged PN. However, the decreased pH due to cysteine can result in an increased risk for metabolic acidosis.^{19,22} Despite providing additional sodium acetate to the CYS group in this study, the CYS group did have a larger anion gap in comparison to the ISO group (Table 1). This result, however, does not appear to be clinically significant and is consistent with previous studies.^{11,16} While it remains unknown, another potential benefit of cysteine supplementation is increased taurine synthesis. Taurine, a conditionally essential amino acid, is important for neurodevelopment and its deficiency has been implicated in the pathogenesis of parenteral nutrition associated liver disease.⁵⁰

Conclusion

In conclusion, when compared to neonates who received isotonic noncysteine PN, high-dose cysteine-HCl supplementation (121 mg/kg/day) for at least 1 week resulted in no change for the group mean of erythrocyte total glutathione, GSH, GSH:GSSG, or FSR-GSH. These results should be taken with some caution considering the smaller than anticipated sample size and heterogeneous nature of the pilot study population. Despite these findings, a larger, positive change in erythrocyte GSH and total glutathione was noted in the group who received cysteine supplementation compared to the control group. In addition, a lower enrollment weight and need for a RBC transfusion were independently associated with a decrease change in GSH and total glutathione. To determine whether cysteine supplementation increases GSH concentrations and decreases neonatal morbidities in

specific populations of critically ill neonates, a substantially larger sample size would be required. Future studies targeting term neonates with respiratory distress and congenital defects may be warranted.

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Abbreviations

GSH	reduced glutathione
GSSG	oxidized glutathione
FSR-GSH	fractional synthetic rate of glutathione
SNAP	score for neonatal acute physiology
PN	parenteral nutrition
CYS	cysteine
ISO	isonitrogenous
RBC	red blood cell
IL-6	interleukin-6
AA	amino acids

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Clinical Relevancy Statement

Improving antioxidant protection and decreasing reactive (per)oxidation and inflammatory species is essential for reducing neonatal morbidity and mortality. This pilot study suggests that high dose parenteral cysteine supplementation in critically neonates results in a larger and more positive individual change in the antioxidant glutathione, and a lower weight or receipt of a red blood cell transfusion is associated with a decrease in the effective change of this antioxidant.

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Figure 1.
Synthesis and utilization of reduced glutathione (GSH) and oxidized glutathione (GSSG).

Figure 2.

Individual change in (infusion day – baseline) for reduced glutathione (GSH), total glutathione (GSH+GSSG), and reduced:oxidized glutathione (GSH:GSSG) in the isonitrogenous noncyst(e)ine (ISO) and cyst(e)ine groups. Results are reported as a mean $\pm$ SEM. *P= .02.

Table 1**Baseline Characteristics**

Characteristics at baseline and infusion day for the cysteine (CYS) and isonitrogenous (ISO) groups. Results are reported as mean $\pm$ SD, median (IQR), or percentage where appropriate. IL-6, interleukin-6; PN, parenteral nutrition; RBC, red blood cell; RDS, respiratory distress syndrome.

	CYS (n=21)	ISO (n=17)	p-value
<i>At enrollment</i>			
Gestational age (weeks)	34 $\pm$ 6	34 $\pm$ 6	0.6
Birth weight (kg)	2.4 $\pm$ 1	2.3 $\pm$ 1	1
Day of life	5 $\pm$ 7	3 $\pm$ 4	0.5
Male sex	71%	82%	0.5
Cesarean section	62%	53%	0.5
Multiple gestation	0%	12%	0.2
SNAP score	14 $\pm$ 4	13 $\pm$ 4	0.09
Fraction of inspired oxygen	0.35 $\pm$ 0.2	0.41 $\pm$ 0.28	
1 minute Apgar	6 $\pm$ 2	7 $\pm$ 2	0.06
5 minute Apgar	7 $\pm$ 2	8 $\pm$ 1	0.1
IL-6 (pg/ml)	85 (55, 603)	37 (18, 172)	0.1
Anion gap	8 $\pm$ 2	9 $\pm$ 3	0.5
Clinical diagnosis			0.9
Respiratory distress syndrome	33%	35%	
Persistent pulmonary hypertension	14%	18%	
Cardiac	33%	24%	
Other	14%	12%	
Chorioamnionitis	19%	18%	1
<i>At Infusion Day</i>			
Adjusted gestational age (weeks)	36 $\pm$ 5	36 $\pm$ 6	0.7
Weight (kg)	2.5 $\pm$ 1	2.4 $\pm$ 1	1
Day of life	14 $\pm$ 8	10 $\pm$ 4	0.07
Fraction of inspired oxygen	0.3 $\pm$ 0.2	0.3 $\pm$ 0.3	0.8
IL-6 (pg/ml)	23 (8, 46)	15 (9, 28)	0.7
Days on study PN (days)	9 $\pm$ 3	7 $\pm$ 0.8	0.002
PN amino acids (g/kg/day)	2.8 $\pm$ 1	2.9 $\pm$ 1	0.5
PN lipids (g/kg/day)	3.1 $\pm$ 1	2.5 $\pm$ 1	0.08
PN calories (kcal/kg/day)	98 $\pm$ 26	91 $\pm$ 24	0.5
Receiving enteral nutrition	19%	13%	1
Received RBC transfusion	76%	53%	0.18
Anion gap	12 $\pm$ 4	9 $\pm$ 4	0.01

Table 2
Erythrocyte Glutathione Concentrations and Synthetic Rates

Erythrocyte total glutathione concentration, reduced glutathione (GSH), reduced:oxidized glutathione (GSH:GSSG), and fractional synthetic rate of glutathione in the cyst(e)ine (CYS) and isonitrogenous (ISO) groups. Results are reported as mean±SEM or median (IQR).

	CYS (n=21)	ISO (n=17)	p-value
Total Glutathione (mmol/L)			
Enrollment	1 (0.7, 1.8)	1.8 (1.4, 2.5)	0.1
Infusion	1.4 (1, 2.7)	1.7 (1.1, 1.9)	0.8
GSH (mmol/L)			
Enrollment	1 (0.6, 1.8)	1.7 (1.3, 2.4)	0.1
Infusion	1.4 (0.9, 2.6)	1.6 (1, 1.8)	0.7
GSH:GSSG			
Enrollment	13 (9, 32)	16 (12, 23)	0.3
Infusion	18 (12, 27)	11 (9, 17)	0.1
FSR-GSH (%/d)	44±5	49±4	0.4

Table 3
Regression Analysis for Glutathione Change

Regression analysis for the individual change (infusion day – baseline) in reduced glutathione (GSH) and total glutathione. PN, parenteral nutrition; RBC, red blood cell.

	GSH		Total Glutathione	
	Coefficient (±SEM)	p-value	Coefficient (±SEM)	p-value
Enrollment weight (kg)	0.2±0.1	0.04	0.2±0.1	0.05
RBC transfusions prior to infusion (Yes/No)	−0.6±0.3	0.03	−0.7±0.3	0.03